Studies on Verticillium malthausei Ware and Mycogone perniciosa Magnus.

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STUDIES ON VERTICILLIUM MALTHOUSEI WARE AND MYCOGONE PERNICIOSA MAGNUS

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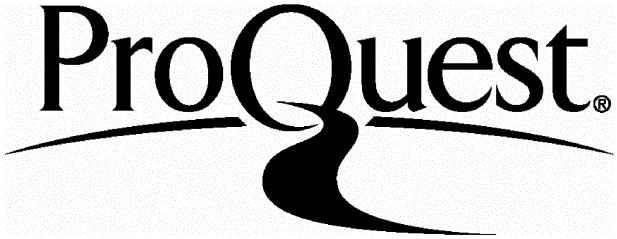

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SUMMARY

The present study mainly concerns *Verticillium malthousei*, a parasite of the cultivated mushroom *Agaricus bisporus*. To a lesser extent, *Mycogone perniciosa*, another pathogen is also examined. After describing the pathogens and disease symptoms, a study was made of the histopathology of diseased mushroom sporophores, resulting in the conclusion that while producing intercellular mycelium, *M. perniciosa* causes host cell breakdown, a condition not observed for *V. malthousei* which prevents normal differentiation in sporophore tissue. A limited host range of both pathogens was indicated by failure to find naturally occurring wild hosts. The significance of spore dispersal of both parasites, from infected mushroom sporophores, on wind-borne dust and debris, by watersplash, flies and contact are discussed in terms of commercial growing practice, the mechanism of mist dispersal being shown to be inoperative for *V. malthousei*. Soil fungistasis, causing the failure of most conidia of *V. malthousei* to germinate in soil, results in the long-term survival of spores of the organism, which is atypical of the genus. The nature of soil fungistasis for *V. malthousei* is shown to be dependant on a requirement of exogenous nutrients for spore germination and the unavailability of such materials in field soil. The low competitive saprophytic ability of *V. malthousei* and susceptibility to soil fungistasis is overcome in soil in the presence of mushroom mycelium,

but infection of sporophore tissue only was observed. The ineffectiveness of dithiocarbamate fungicide treatment in controlling disease caused by V. malthousei is examined in terms of fungal resistance, fungicide stability, toxicity and selectivity. The apparent greater efficiency of benomyl is discussed with respect to possible hazards in its use.

1. INTRODUCTION

The cultivated mushroom Agaricus bisporus (Lge.) Sing. is susceptible to several fungal pathogens of which Verticillium malthousei Ware causing 'dry bubble' and Mycogone perniciosa Magnus producing 'wet bubble' disease are probably the most serious. These diseases, which were for many years confused, can cause considerable loss, varying from 10 - 25% (Constantin and Dufour 1892 b, Lambert 1930) to crops being abandoned during epidemics of the diseases (Veihmeyer 1914). A survey (Last and Gandy 1965) showed that 84% of mushroom farms were infected with M. perniciosa.

a. Historical

The first reported examination of a fungal disease of the cultivated mushroom was by Magnus (1888) who, in the absence of a sexual stage, designated the causal organism Hypomyces perniciosus sp. nov. by analogy with H. chrysogenum (Bull.) Tul. Cooke (1889) reported a mushroom pathogen which resembled Mycogone rosea Link and M. alba Pers. but differed in possessing amber coloured chlamydospores. A disease of cultivated mushrooms was described by Stapf (1889) and attributed to Verticillium agaricinum (Lk.) Corda, the conidial stage of Hypomyces ochraceus Pers. Several Mycogone chlamydospores were seen but were not associated with the pathogen. Prillieux (1892) attributed a mushroom disease to Mycogone rosea Link, which on the imperfect stage only was considered to be synonymous with Hypomyces linkii Tul.

A disease known as 'la mole' was discussed by Constantin and Dufour (1892 a) who noted that the causal organism was similar to

Mycogone cervina. The disease symptoms were divided into two main types - the 'common' form where the stipe, pileus and gills were well defined but swollen and distorted and the 'sclerodermoid' type where little differentiation had taken place, the diseased mushroom having the appearance of a puffball. Two types of spore were found on the 'common' form - long cylindrical, occasionally bicellular 'Verticillium' spores produced in whorls and bicellular Mycogone chlamydospores. This pathogen was referred to as 'Verticillium a grandes spores'. In the sclerodermoid mushroom a Verticillium stage only, producing small unicellular spores, was found and designated 'Verticillium a petites spores'. However, Constantin and Dufour (1892 b), after examination of infected specimens considered that all transitions between the two Verticillium types occurred and assumed the disease symptoms were caused by Mycogone perniciosa Magnus. In 1901 Malthouse described a Verticillium sp. isolated from mushrooms, allied to V. agaricinum, which lacked secondary whorls on the conidiophores. It was thought to be identical with the pathogens described by Magnus (1888), Cooke (1889), Stapf (1889), Constantin and Dufour (1892 a) and Prillieux (1892), the absence of chlamydospores being attributed to environmental conditions.

In America, Veihmeyer (1914) confirmed the two types of diseased mushrooms described by Constantin and Dufour (1892 a) but failed to isolate a small-spored Verticillium species. The pathogen agreed with the description of M. perniciosa Magnus and was isolated from

both 'common' and 'sclerodermoid' (sensu Constantin and Dufour) types of diseased mushrooms. These findings contradicted the results of Constantin and Dufour (1892 a, b), the position being clarified by Smith's demonstration (1924) that sclerodermoid mushrooms could be produced by two distinct pathogens. Infection experiments demonstrated that M. perniciosa Magnus can cause the sclerodermoid condition in mushrooms in addition to the less severe distortions. From other sclerodermoid diseased mushrooms Smith reported a frequently subverticillate isolate producing smaller conidia than M. perniciosa being devoid of chlamydospores. This he designated Cephalosporium Constantini sp. nov. and considered it to be identical with Verticillium a petites spores described by Constantin and Dufour (1892 a). However Smith's description and text-figures indicate a Verticillium sp. and throws doubt on the validity of his generic designation. Smith also described Cephalosporium lamellaecola sp. nov. causing gill fasciation of the cultivated mushroom, which differed from C. Constantini in showing antagonistic effects towards M. perniciosa in mixed culture.

In a detailed study of diseased mushrooms showing similar symptoms to those infected by M. perniciosa, Ware (1933) considered the disease symptoms and description of the causal organism agreed with those reported by Malthouse (1901) and designated the pathogen Verticillium malthousei sp. nov. Ware distinguished his isolates from C. Constantini on the basis of spore size and conidiophore structure but disagreed with Smith (1924) that Verticillium a petites

spores (Constantin and Dufour 1892 a, b) could be referred to C. Constantini and implied from Smith's description and text-figures that the organism was V. malthousei.

Williams (1939) demonstrated that Mycogone rosea is capable of producing a disease of cultivated mushrooms but this disease does not appear to be of commercial importance. Verticillium psalliotae sp. nov. was described by Treschow (1941) which symptomatically differed little from infections produced by V. malthousei. Fassatiova (1965) considered that V. psalliotae Treschow and C. Constantini are indistinguishable from, and can be grouped within, the species V. malthousei on the basis of size and shape of conidia, but these conclusions were drawn from a study of eleven isolates including one of C. Constantini and two of V. psalliotae. Isaac (1967) supports Fassatiova's (1965) contention, emphasising that differences in conidial dimensions and optima of pH and temperature for mycelial growth are poor criteria for speciation in the genus Verticillium.

b. Host Range

While all strains of the commercial mushroom appear to be susceptible, very little information is available on the wild hosts of V. malthousei or M. perniciosa. Constantin (1893a) isolated Mycogone sp. from Amanita rubescens and Inocybe Trinii while Cornu (1881) isolated an organism of the same genus from Amanita praetoria. Mycogone rosea was isolated by Vuillemin (1897) from deformed specimens of Armillaria aurantia and Tricholoma terreum and later Tournais

(1909) reported M. puccinoides from Russula chamaeleontina. Soil has been suggested as the main source of M. perniciosa (Beach 1937, Wood 1960) and V. malthousei (Kneebone and Merek 1961)

c. Cultural Studies

V. malthousei and M. perniciosa grow on most laboratory media used for the cultivation of fungi. Fekete (1967) found that the chemical composition of the medium had little effect on mycelial growth or sporulation of V. malthousei in contrast to Smith's earlier observations with M. perniciosa where the proportion of conidia increased with increasing nitrogen content of growth media. Using unbuffered liquid media Treschow (1941) obtained maximal growth of V. malthousei at pH 5.6 and M. perniciosa at pH 6.7. Illumination appears to have little effect on sporulation in pure cultures of V. malthousei (Fekete 1967) and of M. perniciosa (Smith 1924) while the only effect on the latter of increasing relative humidity was to produce a more flocculent culture (Smith 1924).

Particular attention has been paid to the effect of temperature, to provide a basis for effective methods of control. Minimum, optimum and maximum temperatures quoted for mycelial growth on laboratory media for V. malthousei were 0 - 10, 18 - 24 and 30 - 36 C, for M. perniciosa 10 - 17, 22 - 25 and 28 - 32 C and for Agaricus bisporus 3 - 7.5, 23 - 25 and 29.5 - 30 C (Smith 1924, Lambert 1930, Beach 1937, Treschow 1941, Singer 1961 and Fekete 1967). Thermal death temperatures and times for M. perniciosa were recorded as 46 - 49C for twenty four hours, 42C for six hours, 50 - 55C for four hours and

60 - 65C for two hours while the values were 40C for six hours, 50C for two hours, 48C for one hour and 60C for thirty minutes for V. malthousei (Lambert 1930, Ware 1933, Beach 1937, Anderson 1956, Lambert and Ayers 1957 and Fekete 1967). No survival of either pathogen was found after treatment for twenty four hours with steam and formaldehyde at 49C (Gandy 1954). The values shown for thermal death of these pathogens indicate that neither will survive the second phase of composting (Sinden and Hauser 1953) in which peak heating or pasteurization temperatures of 52 - 60C are maintained for at least 24 - 48 hours.

The optimal temperatures for mycelial growth of the pathogens are similar to that for the cultivated mushroom, but minimal growth temperatures for V. malthousei and Agaricus bisporus are lower than that for M. perniciosa. The temperatures used for sporophore production on a commercial mushroom farm are 15 - 18C. Thus in cropping houses known to be infected with M. perniciosa, a reduction in the cropping temperature to below 13C would be advantageous (Lambert 1930). However this slows sporophore initiation and development to a level commercially unacceptable in this country. Heat treatment of casing material with steam to 82C for twenty minutes or flash pasteurization at 100C to eliminate casing material as a source of infection is often practised on farms (Kneebone and Merek 1961) while steam is often used to raise the temperature to 60C for at least one hour in growing houses at the end of cropping to prevent carry-over and dissemination of disease.

d. Control

The mushroom industry has experimented with chemicals to control fungal diseases. Measures which have been proposed include the disinfection or fumigation of growing houses between crops, the treatment of casing material to kill infective propagules prior to use, treatment of the casing surface with fungicides to prevent infection, the local application of chemicals to infected areas of casing and the general disinfection of the mushroom farm. The uses of fungicidal materials on mushroom farms can be broadly classified into eradicant and prophylactic categories.

Many eradicant materials have been recommended for general hygiene purposes and casing treatment. Constantin and Dufour (1893) suggested the use of 2.0% 'lysol' or sulphur dioxide fumigation to disinfect the interior of cropping houses against Mycogone after emptying, while Malthouse (1901) proposed the use of 0.04 - 0.1% mercuric chloride as a disinfectant spray between crops in infected daves. Veihmeyer (1914) treated cultures of M. perniciosa with formaldehyde and 'coal oil' and the former has since been widely adopted as a fumigant between crops. Solutions of 1 - 2% copper sulphate and carbolic acid (2.5% cresylic acid with soap spreader) were recommended as general disinfectants by Beach (1937) together with fumigation with sulphur dioxide. Beach also suggested treating diseased areas of mushroom beds during cropping with soil impregnated with formalin or with 10 - 15% copper-lime dust. The use of formalin locally on casing was found by Philipp (1963) to completely

inhibit mushroom growth in the application area but Fletcher and Ganney (1969) successfully used 1% formalin during cropping for control of M. perniciosa. Today the main eradicant chemicals used are general disinfectants for internal and external application to growing houses and the mushroom farm site. Formalin is commonly used as an inter-crop fumigant - often together with steam in the 'cooking out' process before emptying a crop. The use of methyl bromide (Hayes and Randle 1968, Hayes 1969) as an end-of-crop fumigant is also growing in popularity.

The use of preventative fungicidal compounds on mushroom beds commenced with the introduction of dithiocarbamate compounds (Sinden and Yoder 1949) which were presumed to possess selective activity against the fungal pathogens. Although good success in disease control was sometimes obtained using zineb (Sinden and Yoder 1949, Yoder, Sinden and Hauser 1950, Zobrist 1953), doubts about its' efficiency were expressed (Yoder et al 1950, Philipp 1963) but over the years since 1949 the recommended application rates for zineb preparations have gradually increased (Kneebone and Merek 1961). A continual search for more effective prophylactic agents has resulted in the use of several other dithiocarbamates e.g. 'Vertomyc' (Fekete and Kuhn 1965, 1966). Today the use of dithiocarbamate fungicides on mushroom beds is widespread - many farms spraying or dusting these materials regularly as part of the normal mushroom growing operation. Ayers and Lambert (1955) proposed the use of chlorinated water (50 - 200 p.p.m. available chlorine) to prevent

bacterial diseases. This procedure also reduced the incidence of Verticillium and Mycogone diseases and the use of chlorinated water has become standard practice on many mushroom farms.

If 'bubble' disease becomes established on mushroom beds, the removal of infected sporophores is widely practised. A serious disadvantage of this method is that workers need to handle diseased specimens - thus creating a dispersal hazard. A method which overcomes this disadvantage is that of physical isolation of diseased sporophores using the isolation cup technique (Jacobs 1965). In this method diseased mushrooms are isolated on the beds by inverting over them a plastic cup which is pushed well down into the casing. This prevents possible spore dispersal by wind, water and insects and enables pickers to work in a diseased area without contaminating their hands or healthy sporophores. The cup is left in place until the end of cropping and is used without fungicides. The application of the control measures outlined above are of little value unless reinforced with good hygiene procedures. Every attempt must be made to reduce the general level of site contamination by infective propagules of V. malthousei and M. perniciosa.

The present work is mainly concerned with a study of selected aspects of the biology, epidemiology and control of V. malthousei. In certain aspects of the work M. perniciosa is also examined. For convenience the work is sectionalised under appropriate headings.

2. MATERIALS AND METHODS

a. Isolation Methods

Isolates of V. malthousei and M. perniciosa from infected mushrooms obtained from Wrington Vale Nurseries Ltd., Somerset, were cultured at 24C on 2% malt extract agar (Oxoid) or on malt agar containing 1 ; 30,000 rose bengal and 30ug/ml streptomycin (Martin 1950) and subsequently maintained on malt agar. V. Malthousei and M. perniciosa isolates degenerate in staled culture necessitating weekly subculturing. To avoid possible degeneration through repeated subculturing, fresh isolations were made approximately every two months.

b. Mushroom Growing Methods

Commercial white strains of the cultivated mushroom Agaricus bisporus were used (viz. 'White Queen 101', 'Somycell 57 and 22', and Darlingtons White). All strains were grown and maintained on malt extract agar (Oxoid) without peptone.

Spawn-run horse manure compost used in this study was mainly prepared by Wrington Vale Nurseries Ltd., using the two phase short composting procedure (Sinden and Hauser 1953). To produce mushroom sporophores in the laboratory the spawn-run compost was packed into four inch 'Hartmann' fibre pots to within 2.5 cm. of the top. Other containers i.e. plastic boxes and glass beakers were used for specific experiments. To induce sporophore initiation the compost was covered with a 2 cm. casing layer of moist sphagnum

peat buffered with limestone chippings (pH 8.0 - 8.3), or a limestone loam soil (pH 7.0 - 7.1) obtained from the University grounds. Both peat and soil were air dried for storage purposes, the latter being passed through a 2 mm. sieve prior to use.

The casing was kept moist during mushroom development by the capillary wick method of Flegg (1962). Before use the nylon felt wicks were boiled for one hour to remove any chemical dressing while between experiments the wicks were thoroughly washed and sterilised by autoclaving. All casing experiments, unless indicated, were carried out in a small growth room maintained at 15 - 18 C and 70 - 95% relative humidity. Under these conditions, mushroom initials appeared in fourteen days, maturing seven days later. Pyrethrin sprays were used periodically to prevent or control fly infestation.

Specific techniques employed in the experimental work are for convenience discussed in the appropriate sections.

3. DESCRIPTIONS, PATHOLOGY AND HOST RANGE

a. Disease Symptoms

V. malthousei can infect the sporophore of the white cultivated mushroom at all stages in its development. Infection of the sporophore initial results in the most severe symptoms, diseased mushrooms appearing as white to pale gray undifferentiated masses of tissue without recognizable stipe or pileus. (Plate 1). These 'sclerodermoid' mushrooms are tough and elastic and remain on mushroom beds without putrefying. Mushrooms infected between pinhead and button stage show differentiation into pileus and stipe but are abnormal in shape. The stipe is thickened expecially at the base and the pileus is small, tilted and misshapen (Plate 2). Strips of tissue peeling back from the top of the stipe are often seen (Plate 3). Lateral infections of the stipe of a mushroom at button or cup stages results in the formation of a longitudinal sunken area which may extend the length of the stalk. These lesions are usually brown in colour or alternatively bear a 'bloom' of grey sporulating mycelium of the pathogen. Localised lateral pileus infections, occurring before the pileus matures retard expansion of the infected area producing harelip deformities (Plate 4). Superficial infections of mature mushrooms produces slightly sunken brown spots on the upper surface of the cap, which develop a grey bloom (Plate 5). Mature sporophores may develop visible gill infection, but often when the lamellae appear healthy, microscopic inspection reveals the presence of the pathogen. Mycelial growth or sporulation of the pathogen was not observed on

Plate No. 1 Mushroom sporophore, infected with V. malthousei, showing sclerodermoid symptoms - an undifferentiated mass of sporophore tissue without recognisable stipe or pileus. (X 2)

Plate No. 2 Mushroom sporophore, infected with V. malthousei showing thickened stipe with small, tilted and mis-shapen pileus. (X 1)

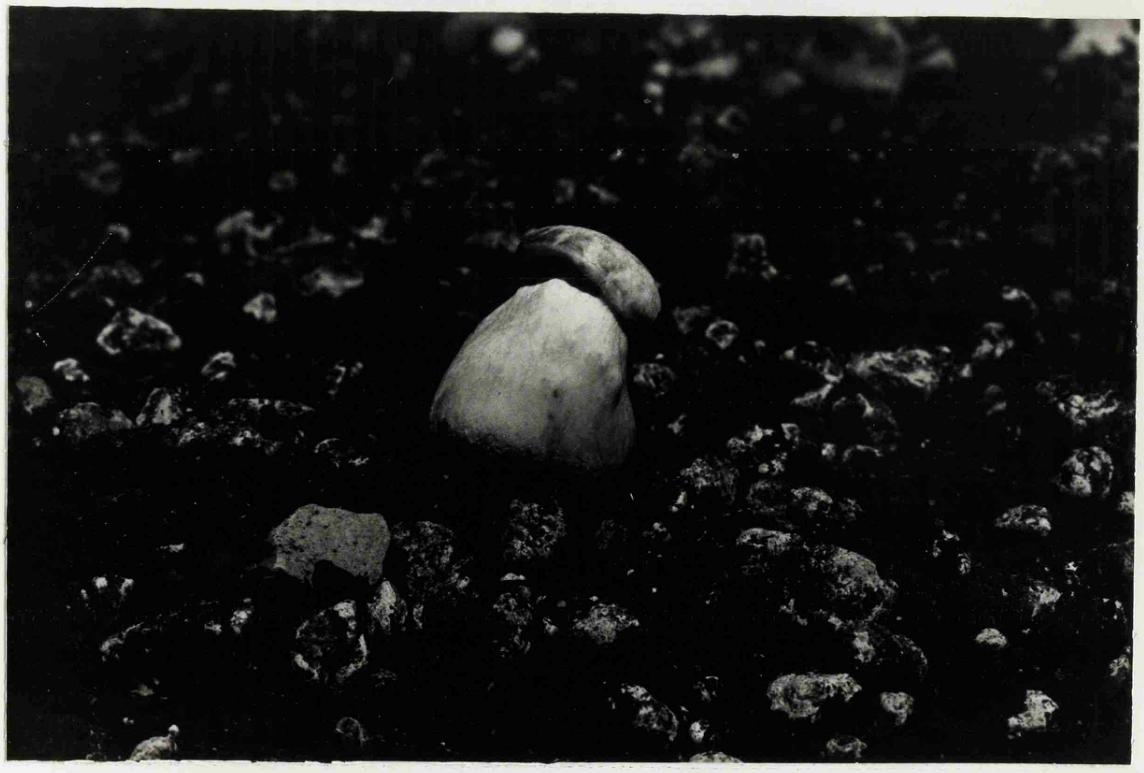
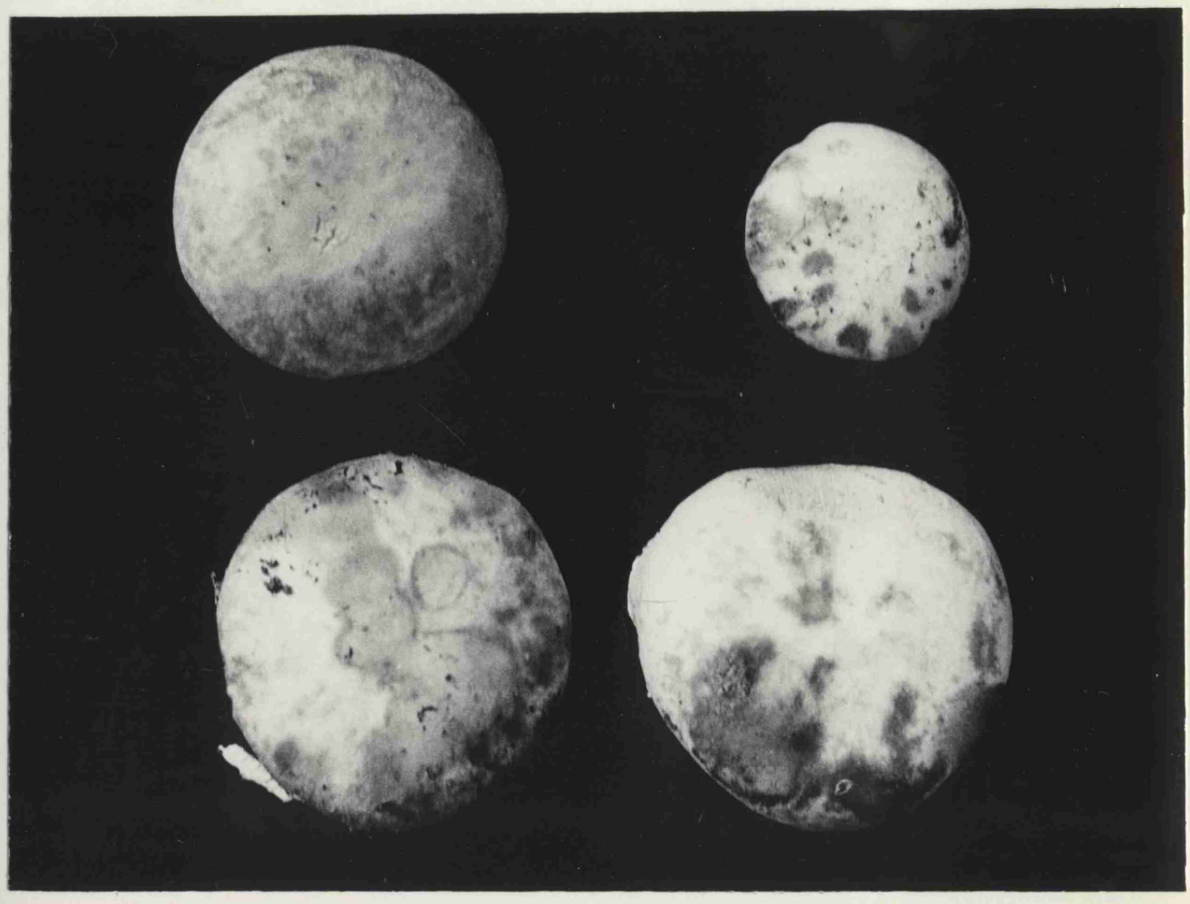


Plate No. 3 Mushroom sporophore, infected with *V. malthousei*, with thickened stipe, small cap syndrome, showing strips of tissue peeling back from the top of the stipe. (X 1.5)

Plate No. 4 Localised lateral pileus infection by V. malthousei, resulting in the formation of a 'harelip' deformity (X 2)

Plate No. 5 Superficial infection by V. malthousei showing slightly sunken brown spots on the upper surface of the pileus, some of which have developed a grey 'bloom' of sporulation of the pathogen. Some bacterial infection is also visible. (X 1)



the casing surface, except on small particles of casing material in contact with the diseased mushroom.

M. perniciosa infection also causes a sclerodermoid deformity in the sporophore of the cultivated mushroom similar to that caused by V. malthousei, but amber beads of liquid exudate are usually seen (Plate 6). Older infected sporophores turn brown, become soft and decompose in several days. Less severely affected specimens are deformed with swollen stipes and small pilei, similar to symptoms produced by V. malthousei. Mature sporophores infected by M. perniciosa bear a dense flocculent white felt of parasitic mycelium on the gills (Plate 7), stipe and also occasionally on the upper surface of the pileus (Plate 8). Hyphal outgrowths from the base of the stipe may colonise the casing surface in the immediate vicinity of the diseased sporophore (Plate 9) to about 1 cm. from the stipe base. The most striking symptomatic difference between infections produced by the two pathogens is the appearance of amber coloured beads of liquid exudate. However this is not an absolute distinguishing feature as similar droplets have been seen occasionally on mushrooms infected with V. malthousei. Reliable diagnosis of either of these fungal diseases of the cultivated mushroom based on symptoms alone is often difficult.

b. Microscopic Structure of the Pathogens

The structure of V. malthousei is illustrated in Plates 10 and 11. In culture, hyaline septate mycelium, 1 - 5 μ in diameter, close to the malt agar surface produces vertical, hyaline, rarely

Plate No. 6 Scleroderma mushrooms produced by infection with *M. perniciosa*. Beads of liquid exudate are visible (Upper photograph X 2
Lower photograph X 1)

Plate No. 7 Mature mushroom sporophores, infected by *M. perniciosa* bearing a felt of parasitic mycelium on the gills, and in the lower photograph, also on the stipe. (X 1)

Plate No. 8 Group of mushrooms infected with *M. perniciosa* including at least one mature sporophore, bearing a dense flocculent white felt of parasitic mycelium on gills, stipe and upper surface of the pileus (X 1)

Plate No. 9 Limited colonisation of the casing by M. perniciosa growing from infected mushroom sporophores.
(Upper photograph X 1, Lower photograph X 1.5)

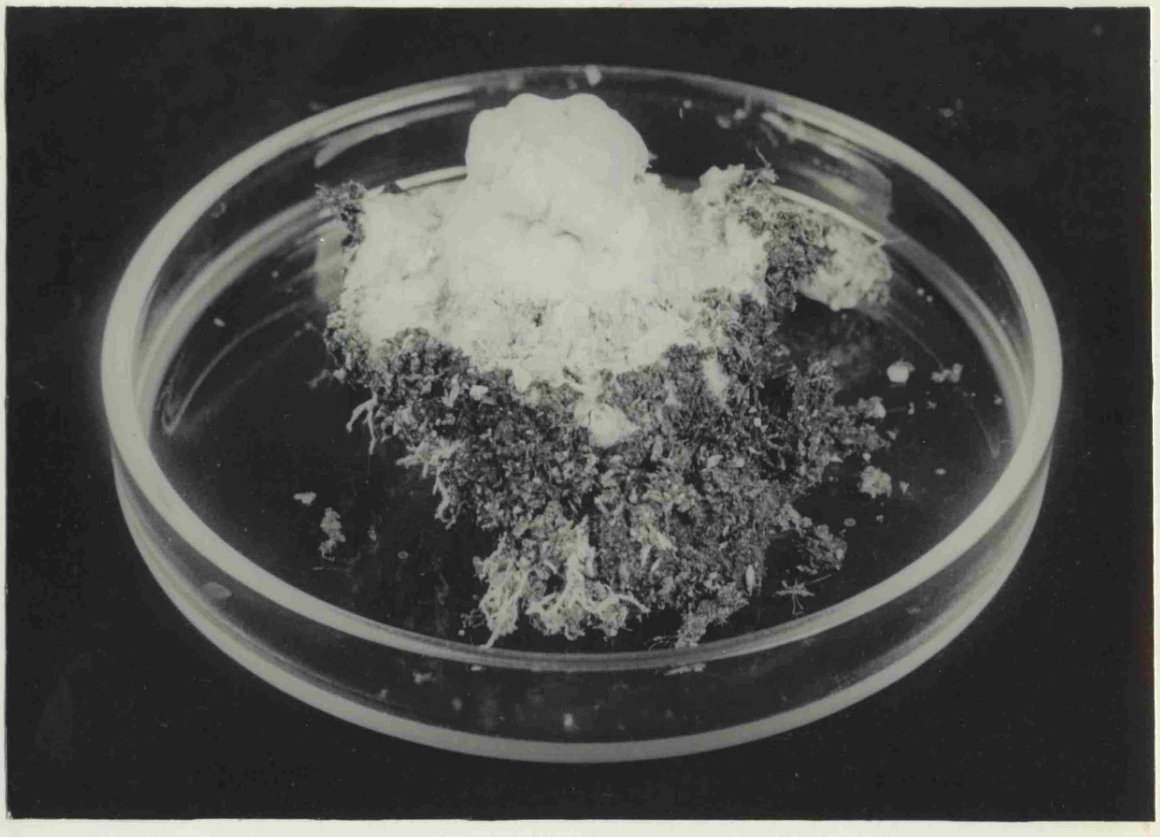


Plate 10 Habit of *V. malthousei* in culture, showing conidiophore, phialides and spore-masses (X 250)

Plate 11 Detail of structure of *V. malthousei* showing mode of attachment of conidia to phialide tip. (Phase contrast X 1000)

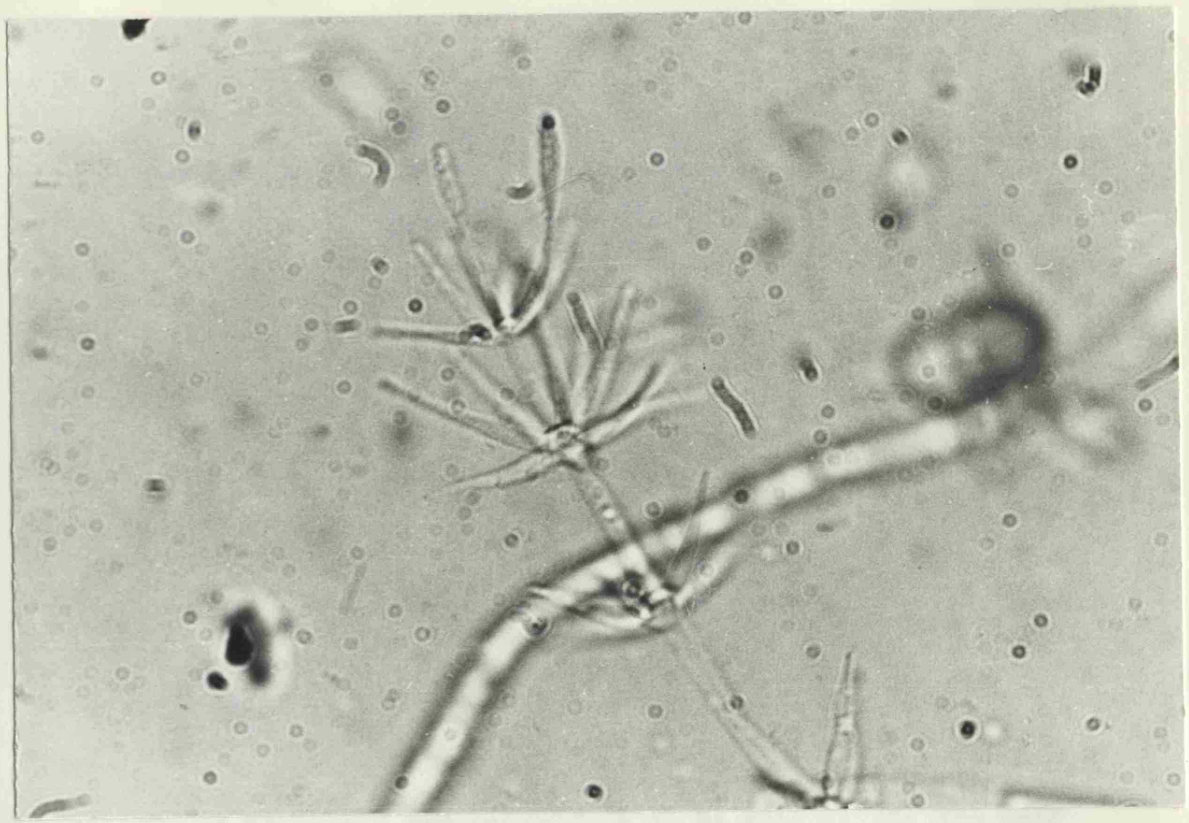
branched conidiophores up to 600 µ in length tapering from 4 µ diameter at the base to 1 µ at the tip, giving the colony a white, compact, smooth, closely-textured appearance. Up to seven whorls of 2 - 9 phialides of 11 - 33 µ in length, of maximum basal diameter 2 µ and tip diameter 0.5 µ, are seen along the conidiophore axis. Hyaline oval to cylindrical non-septate conidia 2 - 12 µ x 1 - 3 µ accumulate in spherical mucilaginous masses at each phialide tip, adjacent spore-masses commonly coalescing. Similar dimensions were recorded from material associated with infected mushrooms. The above description compares very closely with that of Ware (1933) who quotes larger maximum dimensions for conidiophore and phialide length and gives conidial dimensions as 3 - 16 µ x 1.5 - 5.0 µ.

Plates 12 and 13 illustrate the structure of M. perniciosa. Colonies of this organism on malt agar are at first white, then brown with a white margin and of a more fluffy texture than V. malthousei. Initially the septate hyaline mycelium 3 - 4 µ in diameter produces terminally vertical hyaline conidiophores up to 400 µ in length tapering from 4 µ diameter to 1 µ at the tip bearing up to six groups or verticils of up to five branches per group. The upper branches 20 - 40 µ in length bear single, terminal, thin-walled narrow cylindrical bicellular conidia 9 - 23 µ x 2 - 5 µ with both ends tapered or less frequently with a rounded apex. On shorter branches of older mycelium large numbers of single rounded terminal thick-walled, warty, unicellular chlamydospores 13 - 20 µ x 17 - 23 µ are formed. When immature, these are hyaline

Plate 12 Habit of *M. perniciosa* in culture (X 250)

Plate 13 Upper photograph - conidiophores bearing conidia of *M. perniciosa* (X 700)

Lower photograph - chlamydospore of *M. perniciosa* with basal cell attached to parent mycelium (X 1500)



but rapidly turn light brown. Each chlamydospore is produced on a thin-walled hyaline rounded smooth cell 8 - 13 μ x 11 - 18 μ and since chlamydospore and basal cell are usually dispersed as a single unit, the chlamydospores are often described as bicellular. This description agreed closely with that of Constantin and Dufour (1893) and Smith (1924).

One tube culture of M. perniciosa on malt extract agar incubated at 24 C in the dark, produced a white sector with a close woolly texture at the growing edge of an otherwise normal colony. This white variant possessed mycelium, conidiophores and conidia identical in structure and dimensions to M. perniciosa, but only immature hyaline chlamydospores were produced. Successive subcultures of this organism on malt extract agar produced colonies maintaining these characteristics. Infection experiments showed the variant to be infective producing sclerodermoid sporophores identical with typical infected specimens but lacking mature chlamydospores (Plate 14). This mutant was used in survival experiments to be described later.

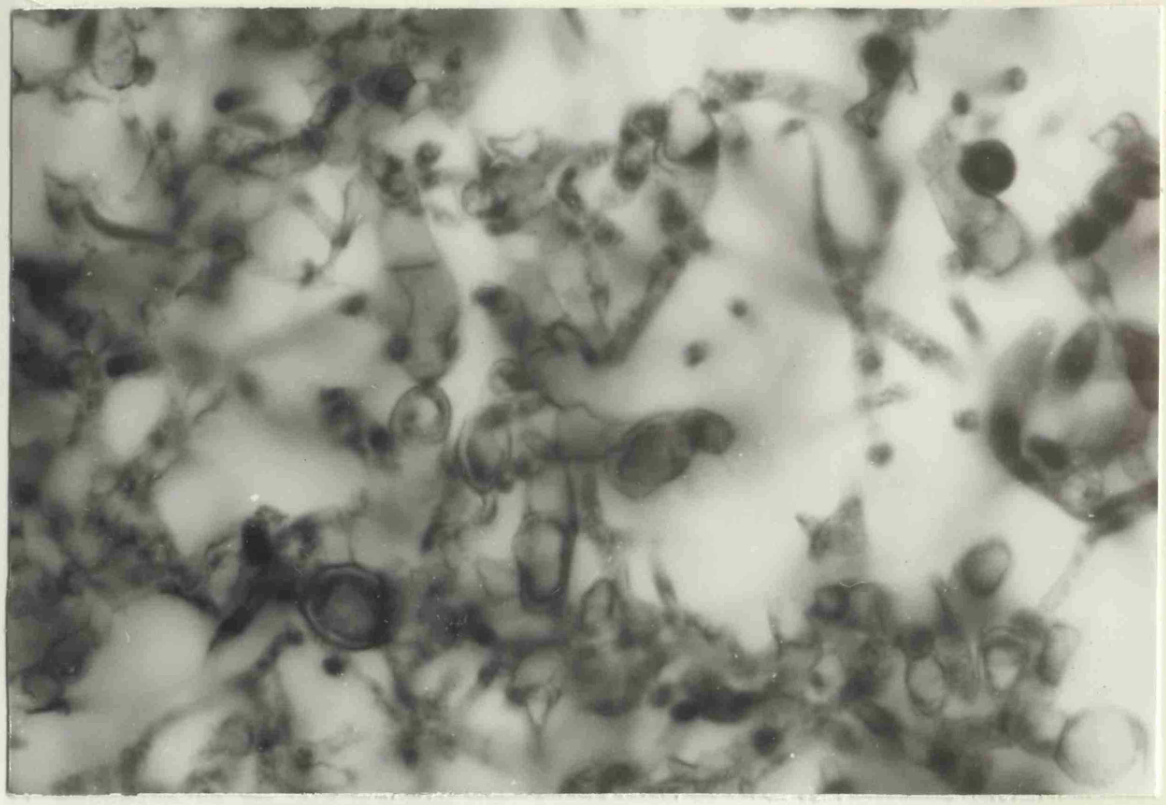
c. Internal Anatomy of Diseased Mushrooms

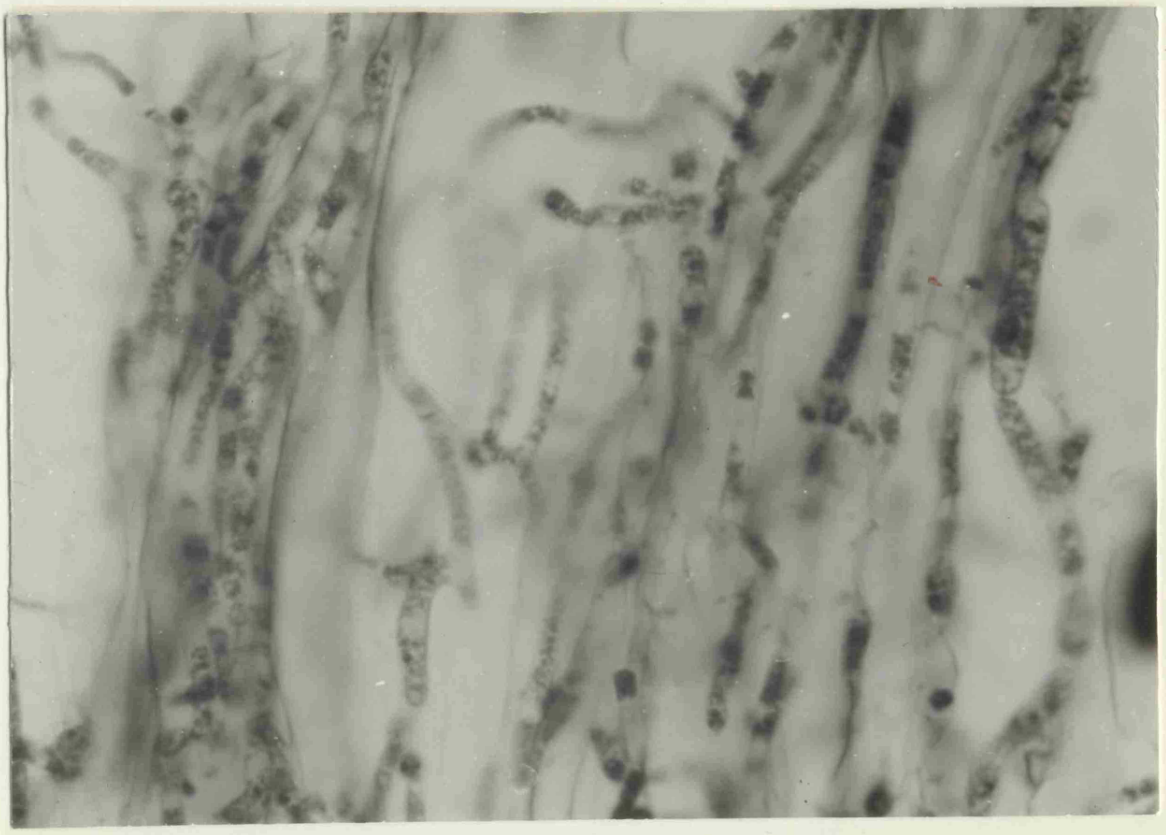
Ware (1933) observed, apart from a narrow discoloured zone below the surface, that the mushrooms infected with V. malthousei were white in colour but possessed a more felt-like consistency than healthy tissue. Sporulation of the pathogen occurred only at the surface of the sporophore while mycelium ramified throughout

Plate 14 Section through sclerodermoid mushroom infected with mutant of *M. perniciosa*.

Upper photograph - superficial region of mutant mycelium with immature chlamydospores (X 700)

Lower photograph - disintegrated host tissue with profuse growth of mutant mycelium (X 1000)





the host. The intimate relationship between the cells of host and pathogen was not described.

Mycelium of M. perniciosa was found at a considerable depth in host tissue by Smith (1924) who described a zonation effect consisting of an outer thick felt-like layer of parasitic hyphae bearing chlamydospores and conidia overlying a band of dead host cells. Beneath superficial layers a zone of extensive parasitic mycelium was observed, which stained densely at the periphery, suggesting extracellular enzymic activity. Few hyphae of the pathogen were seen in the centre of infected mushrooms. The mycelium of M. perniciosa was described as intercellular, in some cases just penetrating the cell walls. In a later study Chaze and Sarazin (1936) described expanded parasitic hyphae which compressed and killed host cells.

To determine the nature of the physical relationship between the cells of host and pathogen, the following methods were used.

Cubes of tissue, of side 0.5 cm. were cut from healthy and diseased mushroom sporophores and fixed in formol acetic alcohol (40% formalin 13 ml, glacial acetic acid 5 ml, 50% ethanol 200 ml) for twenty four hours in a vacuum chamber. After twenty four hours in 70% alcohol the specimens were embedded in 'Paraplast' tissue embedding medium (m.p. 56 - 57 C) using an Elliott automatic tissue processor programmed as follows :-

85% ethanol	7 hours
95% ethanol	8 hours
100% ethanol	2 hours
100% ethanol	2 hours

1 : 1 mixture of 100% ethanol and chloroform	2 hours
Chloroform	½ hour
Chloroform	¾ hour
Wax (Paraplast)	1¼ hour
Wax "	½ hour

Wax blocks were sectioned serially using a Reichert rotary microtome.

An alternative embedding method using Polyester Wax (m.p. 37 - 38 C) was also employed. Fixed tissue was placed in each of 70%, 85%, 95% and two changes of 100% ethanol for twenty four hours at room temperature and embedded in wax after twenty four hours in an equal mixture of absolute ethanol and wax at 38 C. Single sections on glass slides were allowed to air dry at room temperature and dipped in a 1% solution of cellulose nitrate in a 1 : 1 mixture of absolute ethanol and diethyl ether. The same solvent was used to remove the film after the straining and dehydrating procedures had been completed prior to mounting the sections.

Two main staining methods were used although many were tried in an attempt to obtain selective staining. The method used most frequently was the Periodic Acid Schiff technique (Hotchkiss 1948) which stained cell walls of mushroom and pathogen. Cell contents were counterstained blue using 0.1% cotton blue in lactophenol. V. malthousei and M. perniciosa mycelium retained more stain with both methods than the mushroom sporophore cells. Alternatively a method suggested by Steedman (1968 personal communication) in

which the sections were stained for four minutes in an aqueous mixture of Procion red, Chlorantin turquoise and Chlorantin blue was used. Cell walls stained blue and the cytoplasm red, pathogenic mycelium again being differentiated by a greater intensity of staining.

For the best results sections were cut at 15 μ but unfortunately such sections contained few complete mushroom cells. Many of these cells from mushrooms at 'cup' or 'open' stage appeared to be without cell contents, presumably due to the presence of large vacuoles resulting from rapid expansion and water absorption during sporophore maturation. Healthy tissue (Plate 15) showed cells with well stained cell walls, which were mostly elongated and orientated randomly except near the surface of the pileus where they were usually parallel with the surface.

Sections of sporophore tissue from sclerodermoid mushrooms infected with V. malthousei showed normal mushroom cells but no orderly arrangement near the surface. Extensive inter-cellular V. malthousei mycelium was observed (Plate 16) but no evidence of any intracellular mycelium was obtained. No sporulation of V. malthousei was observed within host tissues.

Sporophore tissue sections from sclerodermoid mushrooms infected with M. perniciosa show host cell walls which, although of similar size as healthy cells, stained less strongly and appeared irregular and incomplete in outline. This cell wall damage and the observed rapid decay of infected sporophores is in marked contrast

Plate 15 Section through healthy mushroom sporophore.

Upper photograph - showing relatively uniform orientation of elongated mushroom cells near sporophore surface (X 1000)

Lower photograph - showing more random orientation of larger elongated mushroom cells within the sporophore. (X 1000)

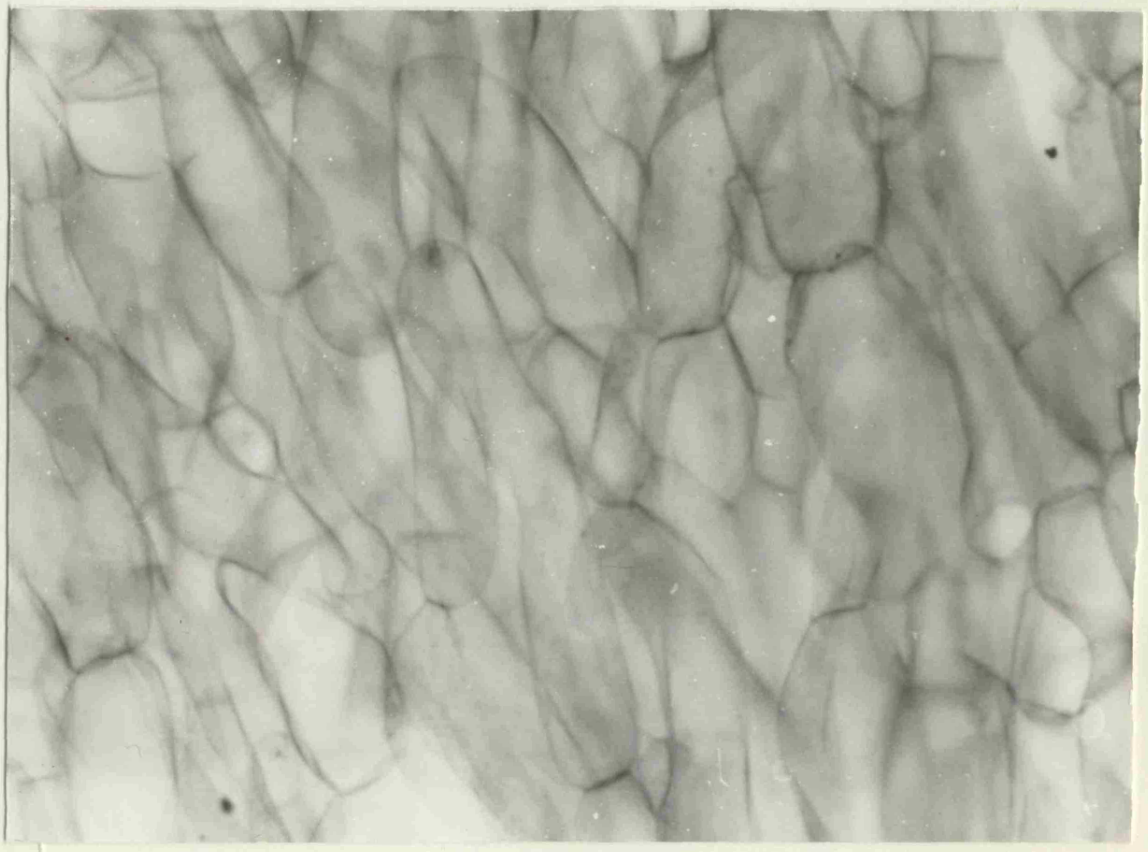
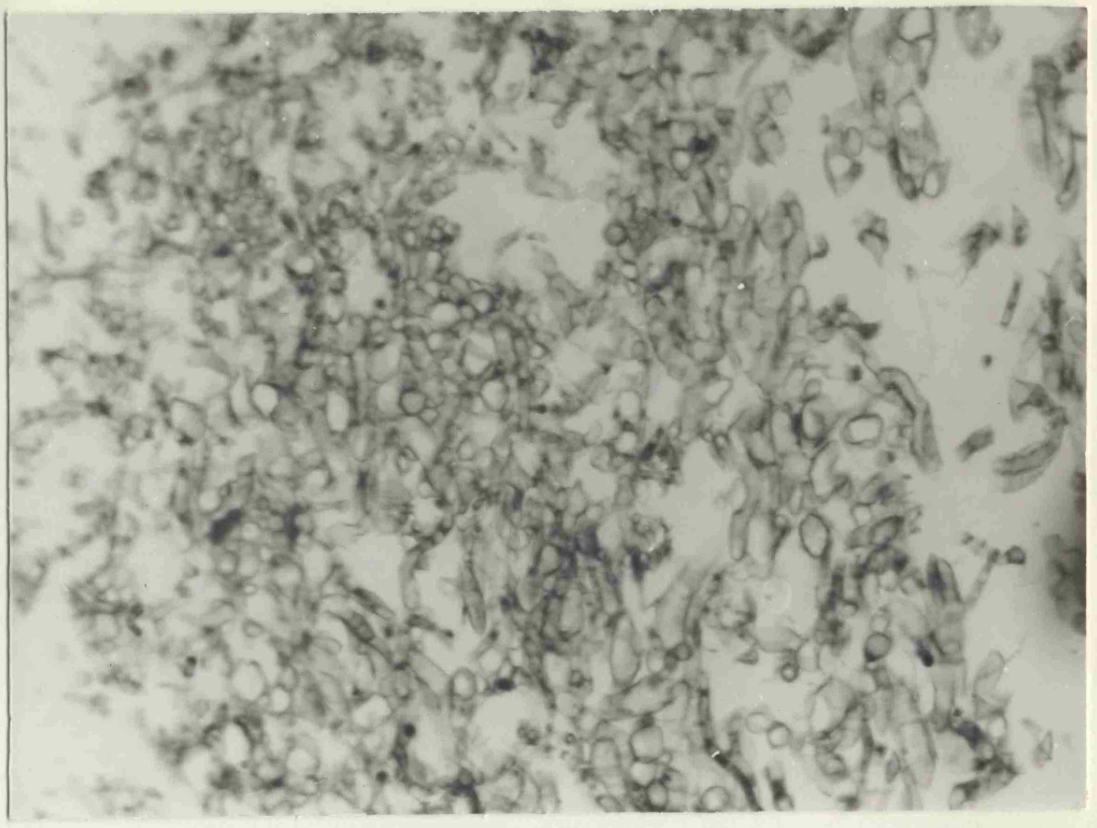
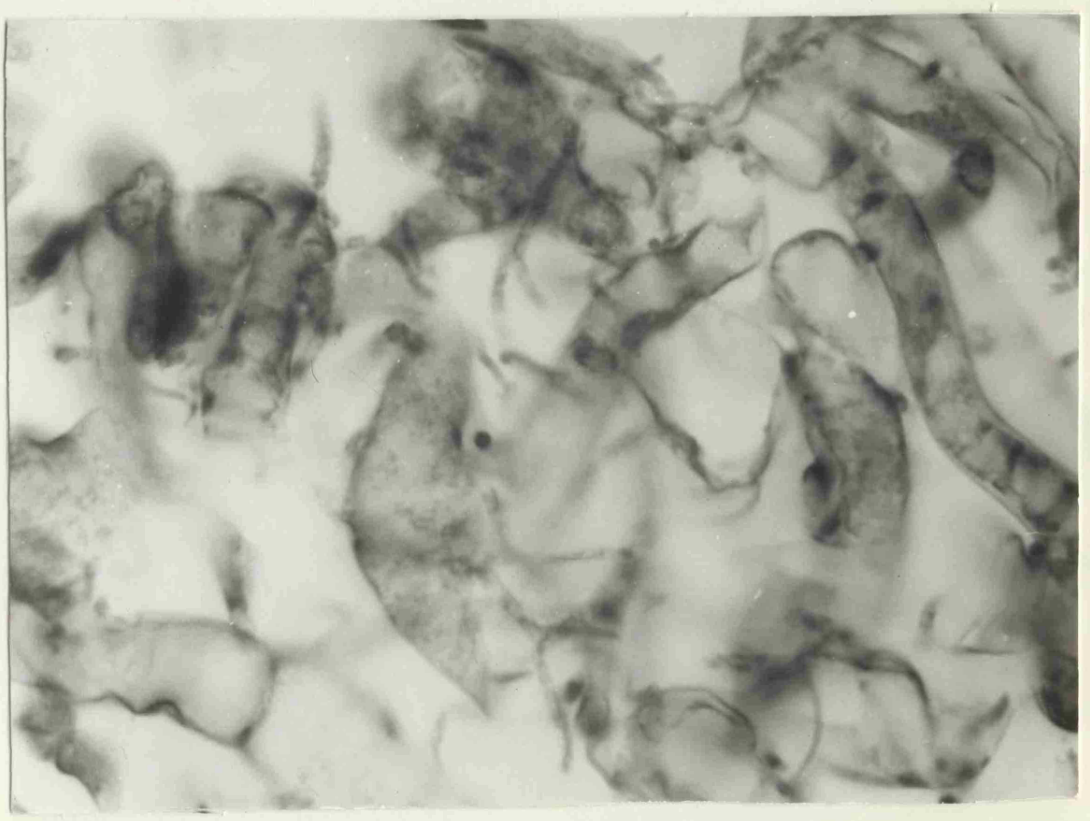


Plate 16 Section through sclerodermoid mushroom sporophore infected with V. malthousei.

Upper photograph - showing well-defined cell walls from tissue adjacent to sporophore surface with no evidence of uniform orientation (X 250)

Lower photograph - V. malthousei mycelium visible in contact with well-defined, disorganised mushroom cells within the sporophore (X 1000)





to the situation in mushrooms infected with V. malthousei where the host cell wall outline remains clear, well-stained and appears undamaged, suggesting that cell breakdown does not occur. Host cells near the surface of the sclerodermoid mushroom infected with M. perniciosa were also disorganized in comparison with the healthy sporophore (Plate 17). Extensive pathogenic mycelium was seen ramifying between host cells but no intracellular mycelium was seen in contrast to the observation by Smith (1924). Production of conidia took place at the mushroom surface but not within diseased tissue. Large numbers of chlamydospores were seen at the mushroom surface and single chlamydospores were observed scattered within the infected mushrooms. The expanded cells of M. perniciosa described by Chaze and Sarazin (1936) were not seen. Two of the zones described by Smith (1924) were observed - the zone of spore production overlying the invaded area of undifferentiated host tissue.

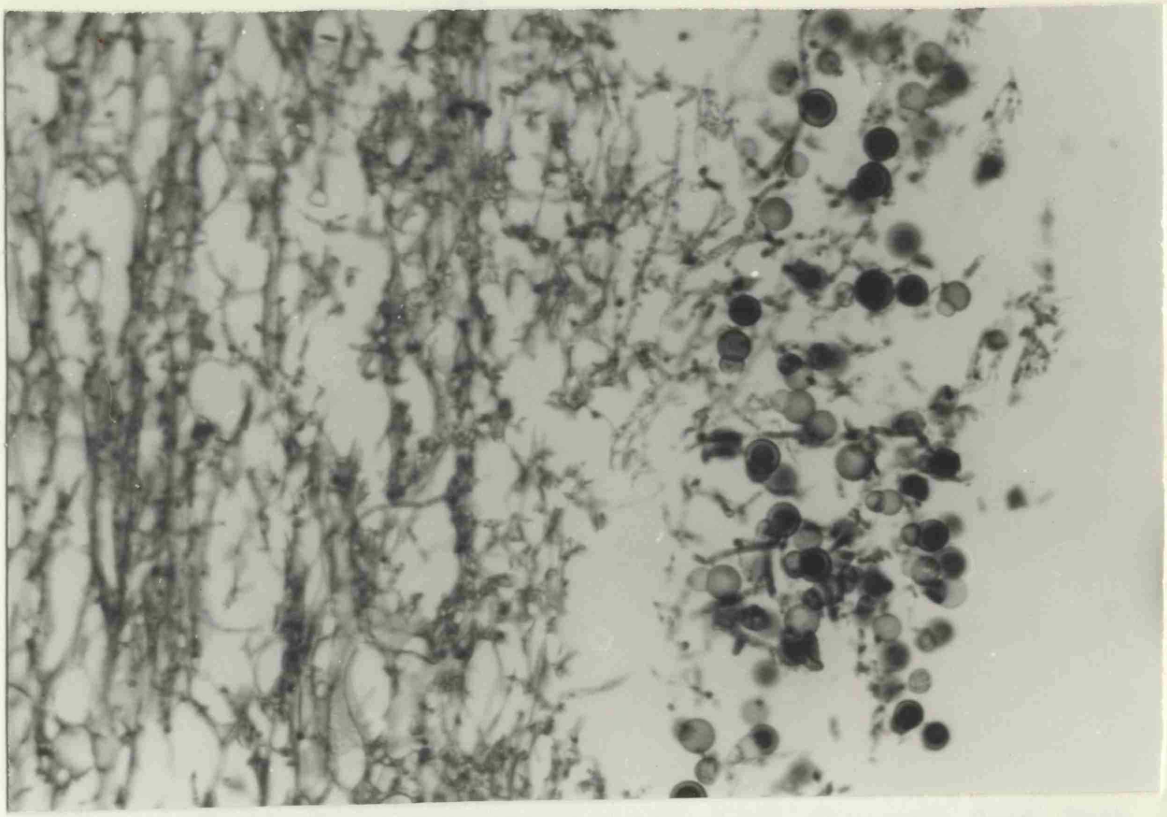
d. Wild Hosts of V. malthousei and M. perniciosa

Since very little information has been published about the natural sources or alternate hosts of V. malthousei and M. perniciosa numerous forays were made in 1965, '66 and '67 to collect a wide range of potential host fungi from a variety of localities including open grassland, grazed pasture, mixed deciduous woodland, coniferous woodland and beech avenues. Some of these locations were near a large mushroom farm where both diseases were endemic. Tissue

Plate 17 Section through sclerodermoid mushroom sporophore infected with *M. perniciosa.*

Upper photograph - showing zone of parasitic mycelium and spore production on the surface of disintegration sporophore tissue (X 250)

Lower photograph - showing damaged mushroom cell walls from within the sporophore with ramifying parasitic mycelium (X 1000)



from specimens suspected of being parasitised was cultured on malt extract agar and sporophores were also incubated in a damp chamber for several days for examination. Direct isolation was attempted where mycelium or spores were seen on the specimens.

Basidiomycete sporophores were collected from the following areas :-

Ashton Gate woods, Gloucestershire
Westonbirt arboretum, Gloucestershire
Hanham woods, Gloucestershire
Longleat Park, Warminster, Wiltshire
Grazed pasture, Combe Down, Bath
Woodland, pasture and open grassland, Claverton Down, Bath
Woodland and open grassland, Bathford, Bath
Grazed pasture, Langford, Somerset
Woodland, Wrington, Somerset
Woodland, Congresbury, Somerset
Woodland, Ascot, Berkshire

The fungi isolated from an extensive range of basidiomycete sporophores included Mycogone rosea Link, Calcarisporium sp., Sepedonium sp. and Chaetomium sp. V. malthousei or M. perniciosa was not observed on or isolated from any of the specimens collected suggesting that these fungi may not parasitise wild basidiomycete sporophores to any great extent.

4. SPORE DISPERSAL

The dispersal of spores of *V. malthousei* and *M. perniciosa* by moving air currents was assumed by Constantin and Dufour (1892 b), Veihmeyer (1914), Beach (1937), Wood (1958, 1960), and Kneebone and Merek (1961). Watersplash spore dissemination was discussed, but not demonstrated, by Ware (1933), Beach (1937), Ayers and Lambert (1955) and Kneebone and Merek (1961). In 1933, Ware reported an association between spores of *V. malthousei* and insects, especially Mycetophilid flies (*Sciara*), the possible role of flies being re-emphasised by Beach (1937), Wood (1958) and Kneebone and Merek (1961). Insect dispersal of *M. perniciosa* spores was noted as a possibility by Constantin and Dufour(1892 b), Veihmeyer(1914), Charles Lambert and Popenoe (1928), Beach(1937), Wood (1960) and Kneebone and Merek (1961). Dissemination of spores of both pathogens by mushroom farm workers, implements or contaminated soil was discussed by Constantin and Dufour (1892 b), Veihmeyer (1914), Beach (1937), Ayers and Lambert (1955) and Kneebone and Merek (1961) while Smith (1924) considered *M. perniciosa* was distributed in infected mushroom spawn. Experimental evidence supporting these views is lacking with the exception of Zoberi's report (1961) of wind dispersal of unspecified spores of *M. perniciosa* from agar cultures and the demonstration by Fekete (1967) of human and mite dispersal of conidia of *V. malthousei*. Dispersal of conidia by airborne mist droplets was reported by Davies (1959) for *Verticillium albo-atrum* but this method has not been examined for *V. malthousei*.

To establish the methods of spore dispersal of V. malthousei and M. perniciosa, wind, watersplash, mist, flies and contact were examined as potential disseminating agents. Dactylium dendroides (Buillard) Fries., the imperfect stage of Hypomyces rosellus Tul. (Petch 1938), which causes the soft mildew or cobweb disease of the cultivated mushroom (Reffstrup 1953, Sinden and Hauser 1954) was included in some of these experiments.

a. Wind Dispersal of Spores

The role of air currents was examined as described by Zoberi (1959). Glass tubes 22 x 1.8 cm. were fitted with a bung at one end, half filled with malt extract agar and sterilised in an autoclave for ten minutes at 10lbs/in^2 in an upright position. After cooling, a second sterile bung was fitted, the tubes being laid horizontally to allow the agar to set. The bungs were removed and the agar strip cut to give a length of 15.5 cm. Sterile non-absorbent cotton wool plugs were fitted and the tubes held for three days at 24 C to check for contamination. Each organism under test was inoculated along the length of the agar strip. After 7 - 14 days of incubation at 24 C each tube was mounted horizontally and connected to a glass impaction chamber (constructed from an 8.5 x 3.5 cm. staining tube with a cut, tapered centrifuge tube), a vertical float-type flowmeter, a screw-clip regulator and Austin diaphragm air pumps (Fig. 1).

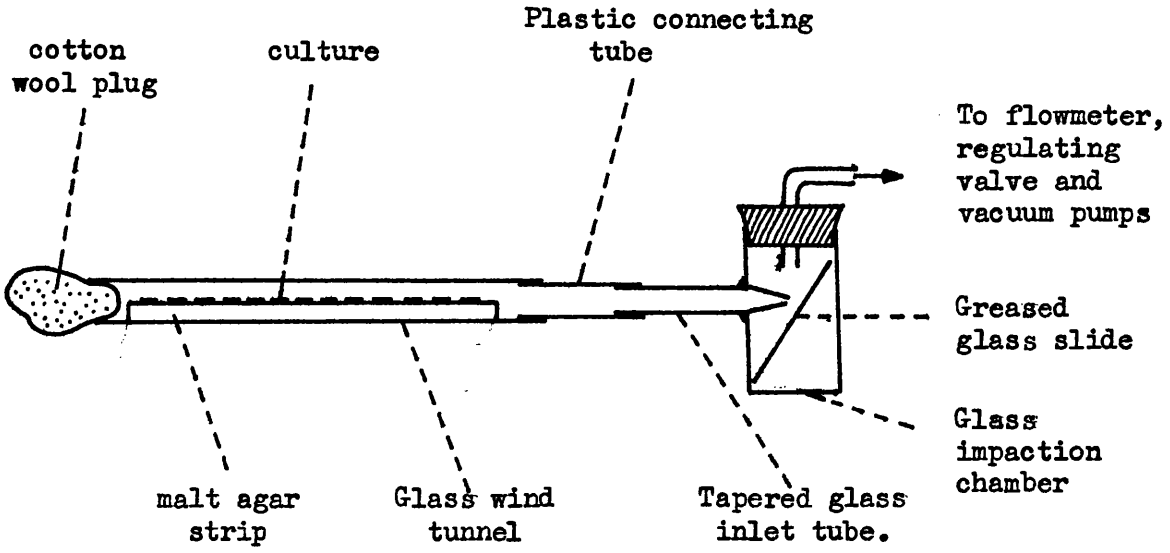


Fig. 1 Diagram of minuature wind tunnel culture tube and impaction chamber

A microscope slide smeared with white paraffin jelly was placed inside the impaction chamber as shown, the air-flow being timed with a stopwatch. After thirty seconds the slide was removed, stained in 0.1% cotton blue in lactophenol and scanned microscopically for the presence of spores. If large numbers of spores were present, the numbers in each of three predetermined microscope fields were counted; otherwise their presence or absence was merely recorded.

Wind dispersal of spores from mushroom tissue was examined by replacing the agar with pieces of tissue of total length 10 cm. cut from the surface of an infected mushroom. It was arranged that the pieces of tissue occupied half the cross-sectional area

of the tube. For D. dendroides 1 cm. lengths of mushroom tissue were used. For these experiments, cotton wool plugs at the inlet end of the specimen tubes were not employed. Cultures and diseased tissue were examined microscopically before and after exposure to the air current to check for spores. When cultures or specimen tubes were changed, the impaction chamber was cleared of spores using several blasts of air. Each treatment was repeated three times using fresh cultures or specimens, all experiments being carried out at 20 - 24 C and 30 - 40% relative humidity.

Nine windspeeds over the range 1.4 - 13.0 m/sec were used with 7-day agar cultures of V. malthousei but no conidia were detected on the slides in any experiment. Chlamydospores were blown from 14-day agar cultures of M. perniciosa by windspeeds of 2.9 m/sec (Table 1) while conidia were dislodged at 5.0 m/sec and above (Table 1). Spores of D. dendroides were removed from 13-day agar cultures by windspeeds of 1.1 m/sec (Table 2).

Windspeeds up to 10.8 m/sec also failed consistently to remove V. malthousei conidia directly from sporophores, but a few spores were found adhering to pieces of peat debris dislodged at higher windspeeds. However, mushroom basidiospores and other spores were frequently trapped. Windspeeds up to 13.0 m/sec were employed for mushrooms infected with M. perniciosa with similar results, conidia and chlamydospores being impacted on the slide only in association with peat particles. Thus while spores are readily dislodged from cultures of M. perniciosa, they are not

Table 1 Spores dispersed by wind from 14-day cultures of M.perniciosa

Windspeed (m/sec.)	Chlamydo-spores	Conidia	Windspeed (m/sec.)	Chlamydo-spores	Conidia
0.7	-	-	0.1	-	-
1.4	-	-	0.3	-	-
2.1	-	-	0.7	-	-
2.9	-	-	1.4	-	-
4.3	+	-	2.9	+	-
5.7	+	-	5.0	+	+
			9.4	+	+

Key + spores observed on slides
- spores absent from slides

Table 2 Numbers of conidia dispersed by wind from 13-day cultures of D. dendroides

Windspeed (m/sec)	No. conidia per 3 microscopic fields									Total
	Slide 1			Slide 2			Slide 3			
0.3	0	0	0	0	0	0	0	0	0	0
0.7	0	0	0	0	0	0	0	0	0	0
1.1	26	0	0	0	0	7	0	2	0	35
1.4	9	0	1	18	20	5	17	14	7	91
1.8	24	6	8	0	15	16	15	9	5	98
2.2	5	7	4	10	0	4	23	29	0	82
2.5	7	5	11	4	5	8	134	93	85	352
2.9	19	11	10	65	57	35	19	26	16	258

removed directly from infected mushrooms by air currents under the conditions described. Fletcher and Ganney (1969) also failed to trap spores of M. perniciosa from an air-stream directed across beds containing infected mushrooms, except in a few instances where spores were removed with debris. The results of similar work with D. dendroides are shown in Table 3, indicating dissemination

Table 3 Numbers of conidia of D. dendroides dispersed by wind from infected mushrooms

Windspeed (m/sec)	No. conidia per field of view			Total
	Slide 1	Slide 2	Slide 3	
0.7	0 0 0	0 0 0	0 0 0	0
1.4	0 0 0	0 0 0	0 0 0	0
2.2	0 3 3	0 4 1	0 2 1	14
2.9	2 1 2	3 1 1	5 15 12	42
3.6	3 4 2	18 14 7	5 0 1	54
6.4	21 31 27	11 6 5	10 8 9	128

of conidia by windspeeds of 2.2 m/sec and above.

Zoberi (1961) found for Trichoderma viride Pers. ex. Fries., which possesses slime spores similar to V. malthousei, that drying of spores facilitates wind dispersal. However for V. malthousei and M. perniciosa, even when infected mushrooms were exposed to a dry air-stream (30% R.H.) at a velocity of 11.6 m/sec for an extended period (thirty minutes), no spores were impacted on the slide except where associated with dislodged debris. Thus the

observation by Zoberi (1961) for T. viride that drying of spores facilitates wind dispersal does not appear to apply to V. malthousei or M. perniciosa.

The effect of wind on cultures of M. perniciosa of varying ages was examined. Tube cultures were incubated for 7, 14 and 35 days and subjected to air velocities of up to 9.4 m/sec. The results of these and repeat experiments (Table 4) showed that the maximum windspeed required to remove chlamydospores from culture decreases as the age of the culture increases, while the effect of culture age on the wind dispersal of conidia is much less marked. This effect could be due to a drying of the cultures or to the basal cell of the chlamydospore breaking away from the mycelium more readily when mature. In all cases where conidia were impacted on slides the numbers were small.

Thus laboratory experiments with miniature wind tunnels indicate that direct removal of V. malthousei and M. perniciosa spores from infected mushroom sporophores in growing houses is not achieved by wind currents. The aerodynamic properties of the apparatus appear satisfactory since large numbers of spores of D. dendroides, mushroom basidiospores and spores of Penicillium sp. (used as a test organism) were impacted on the slide traps. Spores of V. malthousei and M. perniciosa however, can be associated with moist peat and debris blown from infected mushrooms, but only at high windspeeds, far higher than usually encountered in a mushroom growing house, even with forced ventilation. This mode of indirect

Table 4 Wind Dispersal of *M. perniciosa* spores from cultures of varying ages

Culture age (days)	Windspeed (m/sec)	Chlamydo-spores	Conidia	Windspeed (m/sec)	Chlamydo-spores	Conidia
7	0.7	-	-	0.7	-	-
	1.4	-	-	1.4	-	-
	2.9	-	-	2.9	-	-
	5.7	+	-	5.7	+	-
	7.2	+	+	7.2	+	+
	9.4	+	+	9.4	+5	+
14	0.7	-	-	0.7	-	-
	1.4	-	-	1.4	-	-
	2.1	-	-	2.9	+	-
	2.9	-	-	4.7	+	+
	4.3	+	-	7.2	+	+
	5.7	+	-	9.4	+	+
35	0.1	-	-	0.1	-	-
	0.3	-	-	0.3	-	-
	0.7	+	-	0.7	+	-
	2.1	+	-	1.4	+	-
	3.6	+	-	2.9	+	-
	5.0	+	-	5.0	+	-
	5.7	+	+	5.7	+	+
	7.2	+	+	7.2	+	+

Key + Spores impacted on slide

\- No spores observed on slide

wind dispersal is however epidemiologically important, for peat and debris contaminated with conidia of V. malthousei, which has dried out can accumulate in dust on the growing house floor and be disseminated to contaminate the mushroom farm site. Good hygiene is thus necessary to prevent the build-up of windblown dust and debris carrying spores of these pathogens.

b. Watersplash Dispersal of Spores

To investigate the role of watersplash in spore dispersal, eighteen malt agar petri-dishes were arranged around sporulating cultures of V. malthousei, M. Perniciosa or D. dendroides or appropriate infected mushrooms as shown in Figure 2.

Fig. 2 Diagram of arrangement of petri-dishes for watersplash experiments.

After removal of all petri-dish lids, sixty drops of water at the rate of one drop per second were allowed to fall a distance of 75 cm. from a burette held directly above the cultures or infected mushroom sporophores. Following incubation for several days at 24 C the colonies which developed were counted (Tables 5 and 6).

Table 5 Watersplash dispersal of spores from cultures

Plate No. (See Fig. 2)	Numbers of Colonies Developed		
	V. malthousei	M. perniciosa	D. dendroides
1	0	0	0
2	0	0	1
3	4	1	2
4	26	3	12
5	60	14	76
6	136	46	97
7	244	73	90
8	280	62	92
9	249	51	93
10	241	58	113
11	230	63	102
12	282	60	88
13	144	31	125
14	48	15	51
15	4	0	12
16	0	0	0
17	0	0	1
18	0	0	0

Table 6 Watersplash dispersal of spores from infected mushroom sporophores

Plate No. (See Fig. 2)	Numbers of Colonies Developed		
	V. malthousei	M. perniciosa	D. dendroides
1	0	0	0
2	0	3	0
3	1	1	+
4	11	10	+
5	29	23	+
6	84	33	+
7	210	29	+
8	237	+	+
9	244	39	+
10	204	37	+
11	215	26	+
12	198	24	+
13	80	22	+
14	36	2	+
15	6	1	+
16	1	1	+
17	0	0	0
18	0	0	0

Key + Colonies present but not counted due to bacterial contamination.

Fig 3, Plate 18). The results showed the effect of dispersal of spores by watersplash from both cultures and infected mushroom sporophores. The infectivity of such splashed spores was demon-

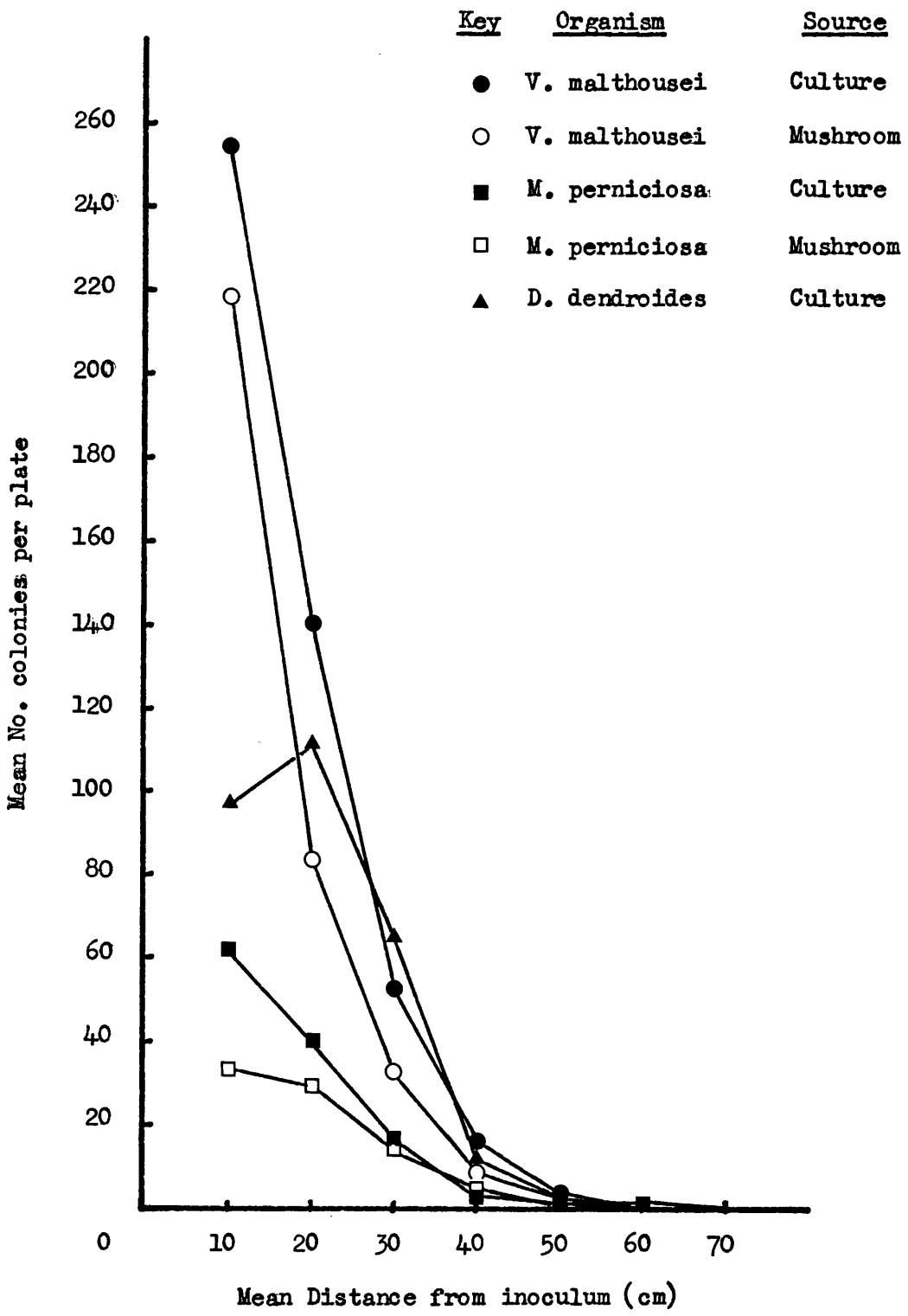
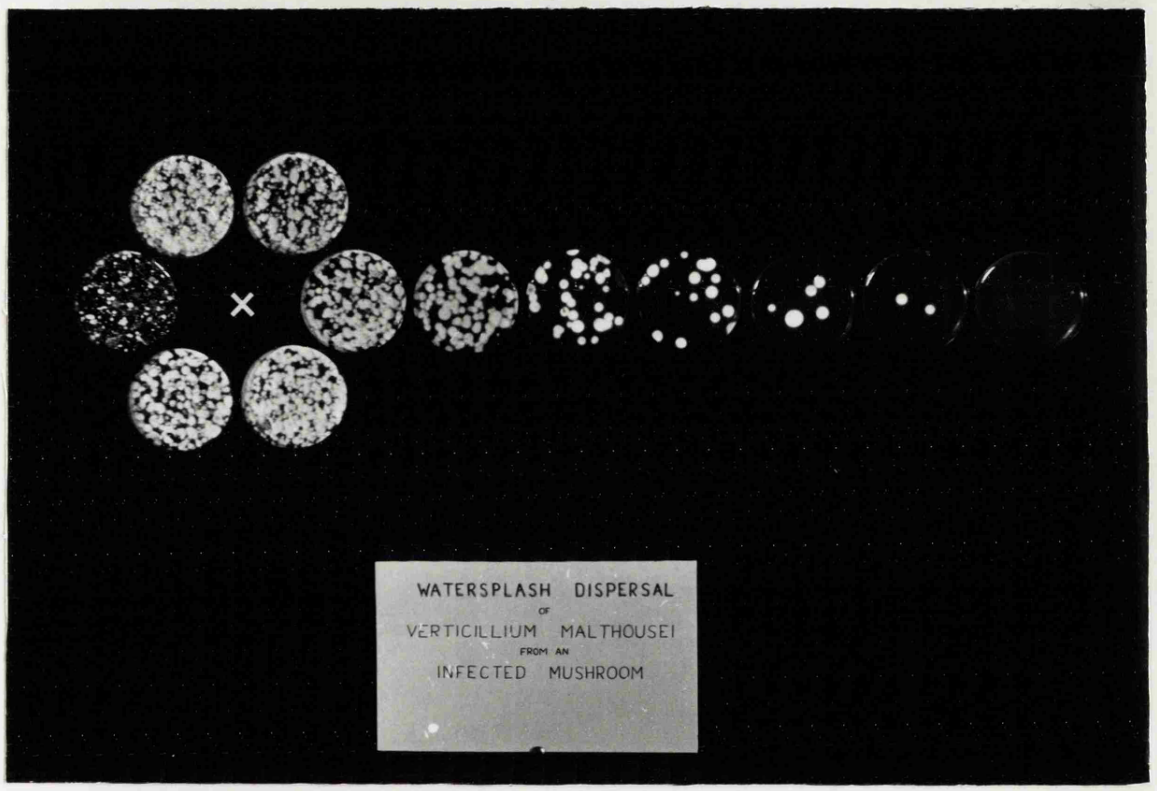


Fig.3 Mean numbers of colonies developed from spores of V. malthousei, M. perniciosa and D. dendroides dispersed by watersplash.

Plate No. 18 Demonstration of colonies growing on malt agar resulting from watersplash dispersal of *V. malthousei* from an infected mushroom sporophore placed at X.


WATERSPLASH DISPERSAL
OF
VERTICILLIUM MALTHOUSEI
FROM AN
INFECTED MUSHROOM

strated under commercial conditions by inoculating a central area of two mushroom boxes each 3 x 2 ft. with spores of V. malthousei. Watersplash dispersal produced large numbers of infected sporophores outside the inoculated area, while only healthy mushrooms were produced on the other box where watersplash dispersal was prevented.

Watersplash is thus a very efficient method of localised dispersal from infected mushrooms for V. malthousei and M. perniciosa. On farms where watering is carried out frequently, watersplash is probably the main method of local dispersal, water droplets being distributed in the main to individual or adjacent trays. However, spores could be splashed to the floor where they could be disseminated much greater distances in association with dust particles. The implications for commercial growing practice are that watering should be done only after diseased mushrooms have been removed or isolated.

c. Dispersal of Spores of V. malthousei by Airborne Water Droplets.

Davies (1959) reported that conidia of Verticillium albo-atrum and Cladosporium sp. were detached and carried by a mist of minute water droplets suspended in a moving airstream passing over cultures. Mist pick-up was considered to differ from the droplet splash mechanism but no clear experimental distinction between these possibilities were made. Dispersal of conidia of Cercosporella herpotrichoides from infected straws by similar airborne water droplets was also demonstrated by Glynne (1953).

To investigate the efficiency of this method for V. malthousei Petenkofer tubes 45 cm. in length, with half the cross-sectioned area of the straight part of the tube filled with malt extract agar, were inoculated at the air inlet end to produce a colony 5 cm long. These cultures were inverted with the air outlet end 10 cm. above the inlet end. After removing the cotton wool plugs, air at a velocity of 2 m/sec was drawn through the tube via a flowmeter and regulation valve by vacuum pump. A water atomizer nozzle was placed near the mouth of the tube and operated for 0.5 seconds, six times into the airstream. In another treatment the atomizer was placed 50 cm. from the mouth of the tube. Control treatments were carried out using an empty atomizer, all tubes being incubated in situ for several days. Water droplet diameters were measured by the method of Rose (1963) and collected for this purpose on a microscope slide covered with soft grease (24% petroleum jelly and 76% liquid paraffin). Care was taken to prevent fragmentation of droplets and a thin layer of liquid paraffin was added rapidly to minimize evaporation. Using a calibrated eyepiece micrometer, the diameters of 100 droplets selected at random were measured, the range of droplet size being 19.3 - 198.4 μ (mean 127 μ) which is within the range 8 - 210 μ recorded by Davies (1959).

In the first treatment a large number of confluent V. malthousei colonies appeared on the uncolonized agar but the numbers of colonies were reduced in the second treatment. In the absence of water droplets, no dispersal occurred. The results are similar to those of

Davies (1959) for V. albo-atrum, but could be explained by large mist droplets dispersing spores by impaction. Water droplets in fog have a mean diameter of 8 μ and a maximum of 22 μ (Keily 1965). Since droplets less than 10 μ diameter will not settle out of air while those over 25 μ consistently do so (Potts 1946), an experiment was designed to examine the effect of droplets of this order of size in a moving airstream on the dispersal of V. malthousei from cultures.

An apparatus was constructed to produce an air suspension of water droplets by aerial condensation (Fig. 4). An oil-free diaphragm pump was used to drive laboratory air through a pre-cooler consisting of 8 m. glass and rubber tubing frozen in ice at approximately -20 C, downwards through the coils of a condenser through which circulated 50% ethanol at -15 C. The air emitted at -10 C was mixed with steam at 100 C in an 8 L glass aspirator submerged in ice and water at 0 C. The resultant condensation produced an aerosol of fine droplets at 20 C. An attempt was made to measure the diameters of these droplets using the paraffin technique of Rose (1963) but complete evaporation took place before the paraffin could be added. Comparing the evaporation rates observed with the smaller of the uncovered droplets obtained from the atomizer it was assumed that the diameters of the evaporated droplets were probably less than 19.3 μ, the smallest size recorded for atomizer droplets. The mist was directed through Petenkofer tubes containing cultures of V. malthousei for five

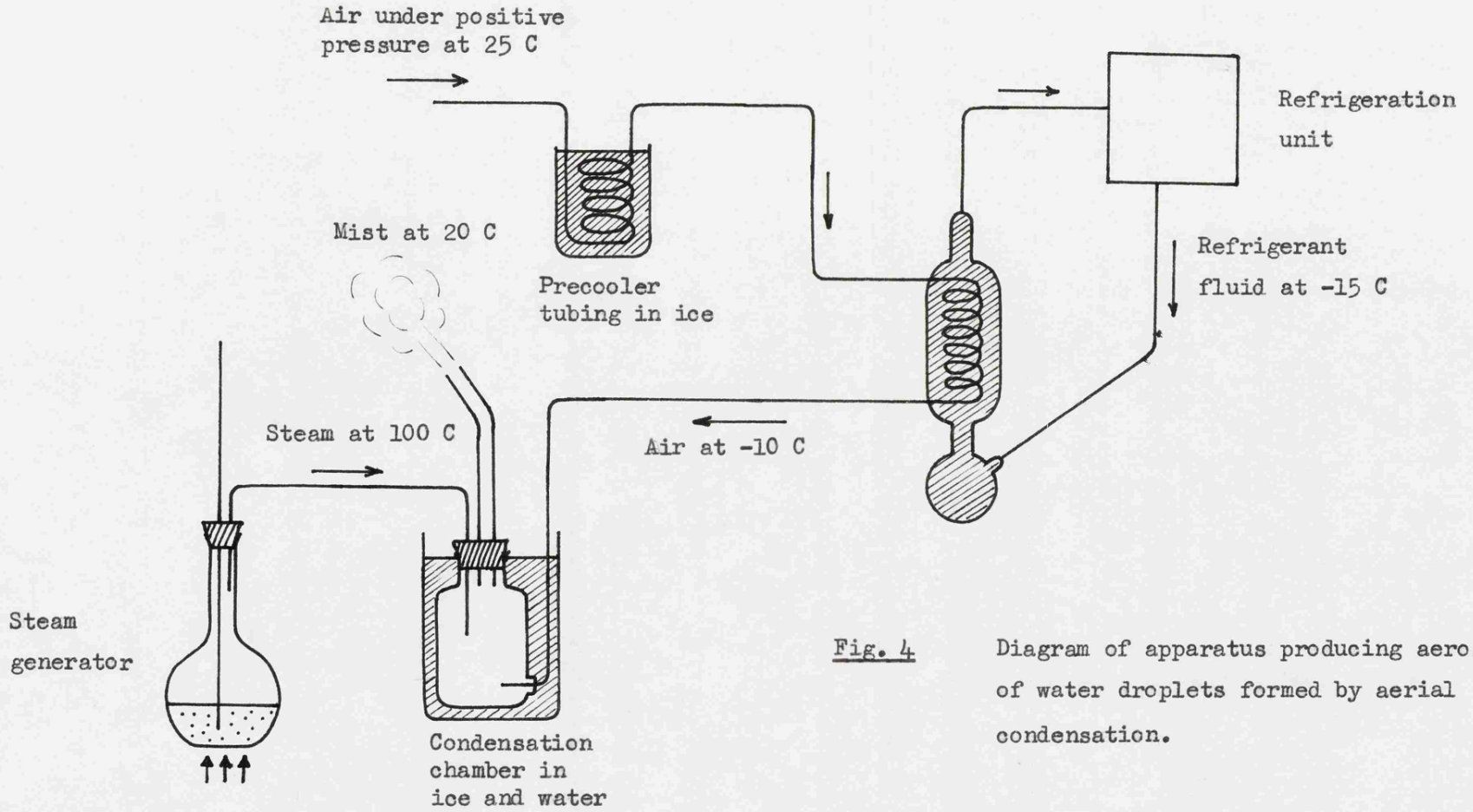


Fig. 4 Diagram of apparatus producing aerosol of water droplets formed by aerial condensation.

and twenty minutes in duplicate and cultures were reincubated in situ as before. A maximum flow-rate over the cultures in the tubes of 0.77 m/sec was achieved with this apparatus. In another similar experiment a seven day plate culture of V. malthousei was held near the wide outlet tube of the condensation chamber allowing the stream of droplets to sweep the colony and to impact on another adjacent dish of malt extract agar. No spores were dislodged by any treatment indicating that true mist dispersal does not operate for V. malthousei, the mechanism of dispersal observed using the larger water droplets produced by the atomizer being probably watersplash.

d. The Role of Flies in Spore Dispersal

Specimens of the mushroom flies Megaselia halterata Wood and Leptocera heteroneura Halliday, collected from Wrington Vale Nurseries, Congresbury, Somerset, were either introduced into malt agar cultures of V. malthousei or M. perniciosa or allowed to alight on freshly picked infected mushrooms. After five minutes, they were removed, stained with 0.1% cotton blue in lactophenol and examined microscopically for the presence of spores. All flies exposed to V. malthousei had conidia adhering to wings, legs, thorax, abdomen and head (Plate19) and following subsequent incubation of flies in malt agar plates for several days at 24 C, many colonies of V. malthousei developed. Chlamydospores and conidia of M. perniciosa were seen on wings and abdomen of

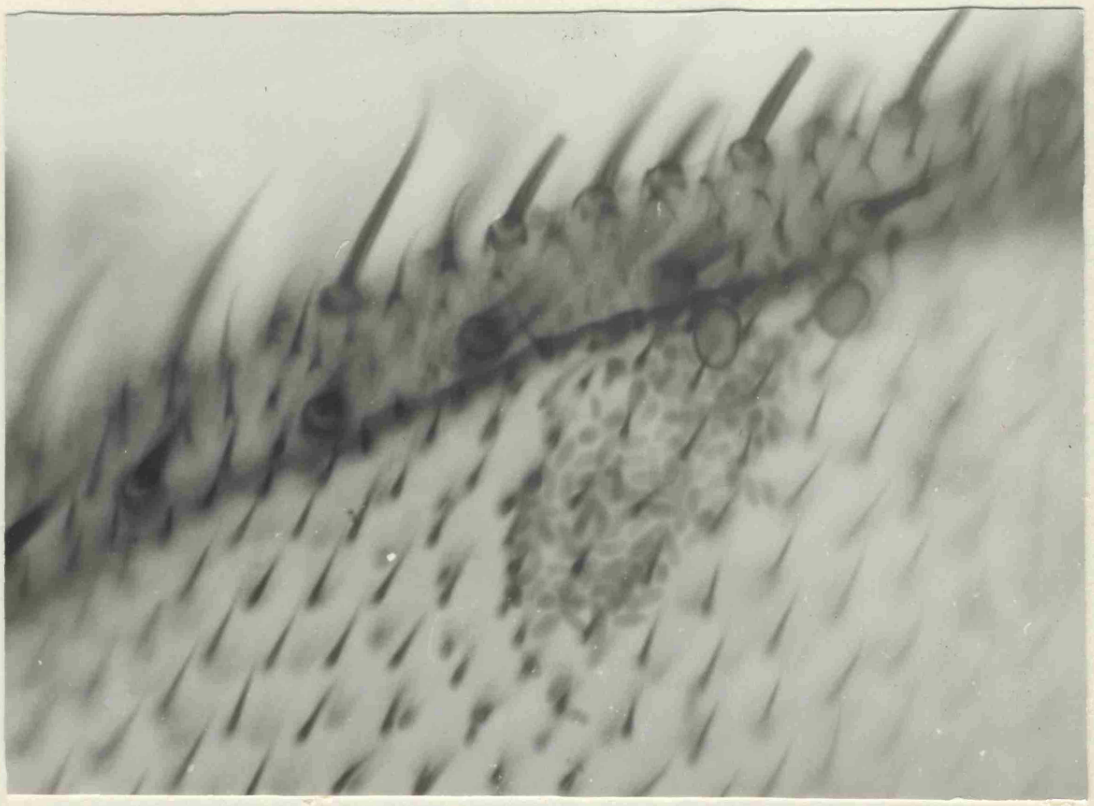
flies associated with cultures (Plate 20) but only conidia were seen on specimens placed on infected mushrooms. The numbers of V. malthousei conidia observed on flies were greater than for M. perniciosa spores.

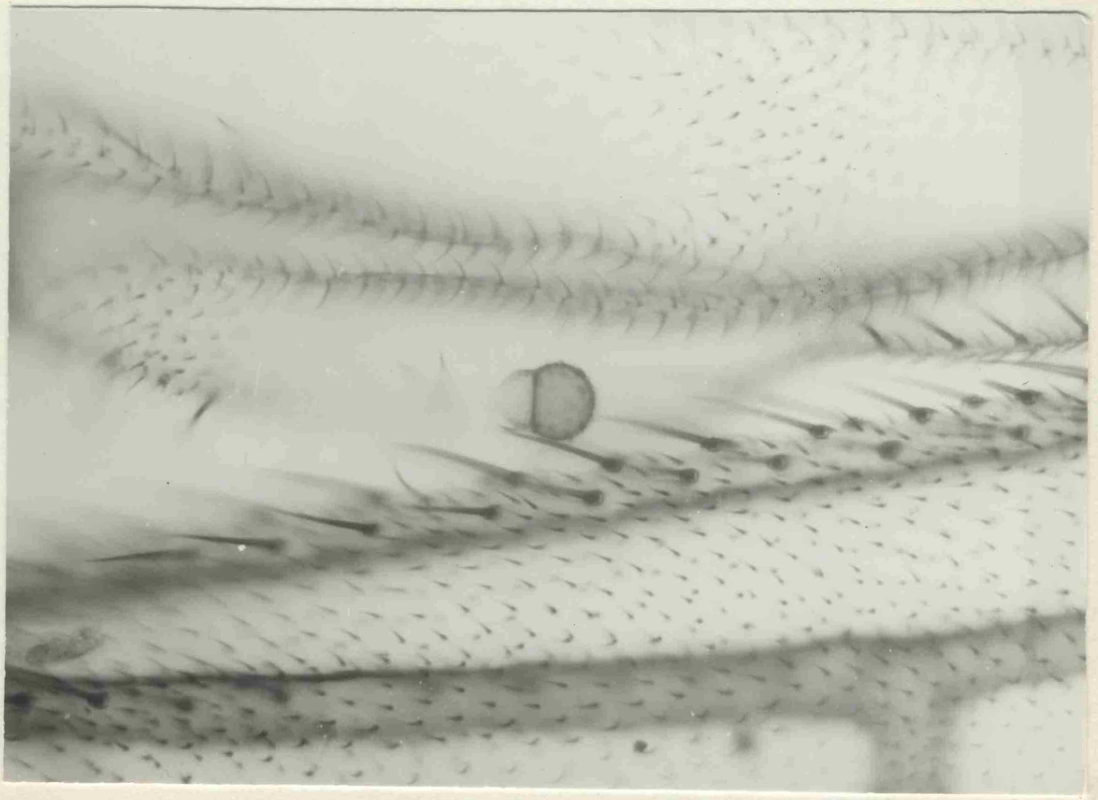
In laboratory experiments flies exposed to diseased and healthy mushrooms in closed containers successfully transmitted infection by V. malthousei and M. perniciosa. Flies collected from a mushroom farm where 'dry bubble' disease was endemic could also transmit V. malthousei to healthy sporophores under laboratory conditions, but flies from growing houses infected with M. perniciosa failed to transmit the disease suggesting that insect transmission is much less efficient for M. perniciosa than for V. malthousei and is possibly a reflection of the relative numbers of spores of the two pathogens previously observed on the bodies of mushroom flies. Spores of the former probably adhere by lodging only whereas conidia of V. malthousei are better adapted to contact dispersal due to the presence of mucilage. Conidia only were observed on flies associated with mushrooms infected with M. perniciosa probably because they are more easily detached than chlamydospores.

The use of fly screens in forced ventilation systems and the proper use of insecticides to control the fly populations on farms is thus essential to control the diseases caused by V. malthousei and M. perniciosa.

Plate 19 *V. malthousei* conidia and mushroom basidiospores adhering to the wing of a mushroom fly (X 1000)

Plate 20 *M. perniciosa* chlamydospore adhering to the wing of a mushroom fly exposed to a culture of the parasite. (X 250)





e. Human Transmission of Spores

Dispersal of spores and infection of mushrooms by this means has long been assumed and has recently been demonstrated for V. malthousei by Fekete (1967). Confirmation of these observations was obtained by making finger imprints of mushrooms infected with V. malthousei or M. perniciosa, on quadrants of 25 dishes of malt extract agar by touching them serially. After incubation for several days, although some colonies of M. perniciosa were heavily contaminated with bacteria, all 100 imprinted areas produced colonies of V. malthousei or M. perniciosa. Thus good standards of hygiene on the part of mushroom farm workers are essential to achieve control of these diseases.

5. BEHAVIOUR IN CASING MATERIALS

Comparatively little detailed work has been done on the behaviour in the casing of V. malthousei and M. perniciosa. Constantin (1892b) observed the reoccurrence of 'la mole' disease in a disused mushroom cave left idle for three years and attributes this to the survival of M. perniciosa within the cave. However, precautions to prevent recontamination were not recorded. Veihmeyer (1914) demonstrated that compost and casing from a growing house badly infected with M. perniciosa, exposed to the weather for five years, failed to induce disease when mixed with fresh compost. For V. malthousei, Beach (1937) reported that cultures on agar media lost their viability through desiccation in approximately six months, while Fekete (1967) showed that conidia did not survive seven months dry storage. While studies on spore germination of both organisms have been made in pure culture (Smith 1924, Ware 1933, Chaze and Sarazin 1936, Fekete 1967) and sterilised soil (Smith 1924, Treschow 1941), no observations have been made on the germination and growth of these organisms through untreated soil or peat.

The method most frequently used by previous investigators for experimental inoculation of mushrooms with V. malthousei was to seed the surface of the casing with a spore suspension (Ware 1933, Ayers and Lambert 1955, Poppe 1967) - a method which produced the full range of symptoms. Contacting the pileus of a healthy mushroom

with a diseased sporophore produced spotting symptoms only (Ware 1933, Treschow 1941, Fekete 1967). Experimental infection of mushrooms by M. perniciosa was usually obtained by sprinkling a spore suspension of the pathogen on the casing surface (Smith 1924, Lambert 1930, Ayers and Lambert 1955, Garibova 1968, Fletcher and Ganney 1969). Under commercial conditions, contaminated mushroom spawn was believed by Veihmeyer (1914) to be the major source of infection, M. perniciosa subsequently growing up through the casing with mushroom mycelium to infect the young sporophores. However parasitic mycelium could not be isolated from spawn-run compost. This was confirmed by Smith (1924) who found no evidence of the presence of M. perniciosa below the level of the soil casing in infected beds except in one instance where a heavy inoculation of spores had been placed in the lower layers of the casing. From these observations Smith (1924) suggested that M. perniciosa attacked the young mushrooms from the casing soil and that the time of infection determined the type or severity of the symptoms. These observations were supported by Fletcher and Ganney (1969) who noted an interval of approximately thirteen days between inoculation and symptom production. It is interesting to note that the brown stains of the cultivated mushroom are not considered susceptible to infection by M. perniciosa (Garibova 1968).

Experiments described below were carried out to investigate the behaviour of V. malthousei and M. perniciosa in soil and peat casing materials. The work is concerned with survival, spore

germination, mycelial growth and mushroom infection.

a. Survival of Spores

Spore survival for V. malthousei was determined in casing materials in relation to their subsequent capacity to infect mushrooms. The mean maximum water-holding capacities of peat and soil casings was found to be 116% and 45% respectively using the method of Keen and Raczkowski (1921). Air-dried peat and soil casings were made up to 50% of their maximum water-holding capacity with a suspension of conidia from 7-day malt extract agar cultures of V. malthousei. A haemocytometer was used to determine the concentrations of spore suspensions and the final numbers of spores in casing materials were 4.87×10^6 per g air-dried peat and 2.73×10^6 per g air-dried soil. Inoculated peat and soil (250 g and 500 g respectively) were placed in thirty unperforated plastic flower pots equipped with loose-fitting lids and were stored in the growth room at 15 - 18 C and 70 - 95% relative humidity. Control treatments consisting of thirty pots each of peat and soil made up to the same water content with distilled water were stored in a similar manner. Water loss was made good by periodically bringing the pots back to their original weight by adding distilled water. To check the viability of spores added to peat and soil, a portion of the spore suspension was incubated for fifteen hours at 24 C on malt extract agar and using the method described later, the percentage germination was found to be 93.6%.

At intervals, two pots from each treatment were removed and used to case spawn-run compost in ten fibre flower pots in the growth room. Moisture was added to the casings by the capillary watering system and the numbers of pots producing only healthy or diseased mushrooms or both was recorded, (Table 7), together with the type of symptoms produced (Table 8). Uninoculated control treatments grew only healthy mushrooms except on two separate occasions when a single mushroom was superficially infected.

The results demonstrate that V. malthousei conidia remain viable and infective in moist casing materials for a considerable period - at least ninety three weeks in peat and seventy eight weeks in soil under the conditions of the experiment, the differences being possibly due to the different storage media or to the greater number of spores inoculated into the peat. High initial numbers of conidia in the casing results in the sclerodermoid syndrome, which gives way to the small cap, swollen stipe and finally cap-spotting or harelip deformities as the number of viable spores is reduced.

Using 4.1×10^4 chlamydospores and 4.0×10^3 conidia of M. perniciosa per g dry weight of peat casing material or 9.7×10^4 conidia only from the mutant, similar infection assays were carried out in a cabinet in the laboratory at 20 - 25 C, over a twenty four week period (Table 9). Control pots produced only healthy mushrooms. Mixtures of M. perniciosa spores survive at least twenty four weeks but conidia from the mutant form could not

Table 7 Numbers of pots producing diseased or healthy mushrooms in casing material inoculated with V. malthousei conidia

Survival period (weeks)	Peat Casing			Soil Casing		
	Diseased mushrooms only	Diseased and healthy mushrooms	Healthy mushrooms only	Diseased mushrooms only	Diseased and healthy mushrooms	Healthy mushrooms only
0	10	-	-	10	-	-
1	10	-	-	10	-	-
2	10	-	-	10	-	-
4	10	-	-	10	-	-
7	10	-	-	10	-	-
21	8	2	-	9	1	-
42	4	6	-	8	2	-
55	6	4	-	10	-	-
78	5	2	3	4	1	5
87	2	2	6	-	-	10
93	3	1	6	-	-	10

Table 8 Numbers of pots with various types of disease symptoms in casing material inoculated with V. malthousei conidia

Survival time (weeks)	Peat Casing			Soil Casing		
	Sclerod-ermoid	Small cap swollen stipe	Cap spotting or harelip	Solerod-ermoid	Small cap swollen stipe	Cap spotting or harelip
0	10	-	-	10	-	-
1	10	-	-	10	-	-
2	10	-	-	10	-	-
4	10	-	-	10	-	-
7	10	1	-	10	1	2
21	10	-	-	10	-	-
42	10	1	2	8	1	7
55	2	4	6	3	-	10
78	4	5	2	1	4	2
87	2	-	2	-	-	-
93	3	-	1	-	-	-

infect mushrooms by week twenty four. Since conidia of parental and mutant cultures probably have similar survival potential, the long-term survival of M. perniciosa is thus via the chlamydospore.

Table 9 Numbers of pots producing diseased or healthy mushrooms with casing material inoculated with M. perniciosa or mutant

Survival period (weeks)	M. perniciosa		
	Diseased mushrooms only	Diseased and healthy mushrooms	Healthy mushrooms only
0	4	1	-
9	5	-	-
15	4	1	-
24	4	1	-

Survival period (weeks)	Mutant form.		
	Diseased mushrooms only	Diseased and healthy mushrooms	Healthy Mushrooms only
0	5	-	-
9	2	1	2
15	-	2	3
24	-	-	5

b. Germination of Spores

(i) General Methods

Spores were harvested from 7-day malt extract agar cultures grown at 24 C by shaking with water and glass beads or by using jets of water from a pipette, any mycelium being removed by filtration through glass wool. When required, spores were washed by centrifugation at 3000 rpm. for five minutes, resuspended in distilled water and recentrifuged, the final suspension being adjusted with distilled water to a concentration of 5×10^5 spores per ml. If the spores were to be mixed with other solutions, appropriate higher numbers were used to give the same final concentration.

All glassware used was washed, soaked in 'Decon' for twelve to twenty four hours, given several rinses in tap, then distilled water and dried in an oven. Microscope slides were boiled in 'Decon' for thirty minutes and similarly rinsed, dried and stored. Before use, the slides were washed in running tap water for several hours, rinsed in several changes of distilled water and air-dried in an oven.

Incubation chambers were constructed from crystallising dishes placed over inverted petri-dish lids containing moist filter paper. One droplet of approximately 0.03 ml. spore suspension was placed at each end of a glass microscope slide supported on a glass 'U' tube. After sealing the chambers with water the spores were incubated at 20 - 24 C for fifteen to twenty four hours. Alternatively 0.03 ml. spore suspension was placed on each of two discs of agar

medium 7.5 mm. diameter and 1.5 mm. thick on the glass slide. After incubation the spores were stained with 0.01% cotton blue in lactophenol and the percentage germination determined for at least two hundred spores in random fields of view (approximately one hundred from each replicate). Germination was considered to have occurred if a germ tube of length at least equal to half the minimum dimension of the spore had been produced. Percentage germination values were not corrected to 100% controls unless stated.

To examine germination in casing materials, the method of Cholodny (1930) was used in which washed spore suspensions were sprayed on glass slides using an atomizer or placed on the slide in droplets which were spread out using an inoculation wire. The slides were then buried vertically or horizontally in casing material. For subsequent examination, the slides were carefully removed, the reverse side cleaned and placed over a warm microscope lamp to dry. Larger soil particles were removed using fine forceps or a needle and percentage germination values determined as before using a stained preparation or using positive or negative phase contrast microscopy as appropriate.

Germination of V. malthousei conidia in soil was also examined on filter membranes using the method of Adams (1967). Oxoid cellulose acetate membranes were placed in filter holders and an unwashed spore suspension was added and filtered. If required the spores were washed by passing distilled water through the membrane, which was then buried in casing material. Later after removal, the membranes

were stained in 0.01% cotton blue in lactophenol, cut into strips 2 cm. wide and mounted in glycerol for microscopic examination.

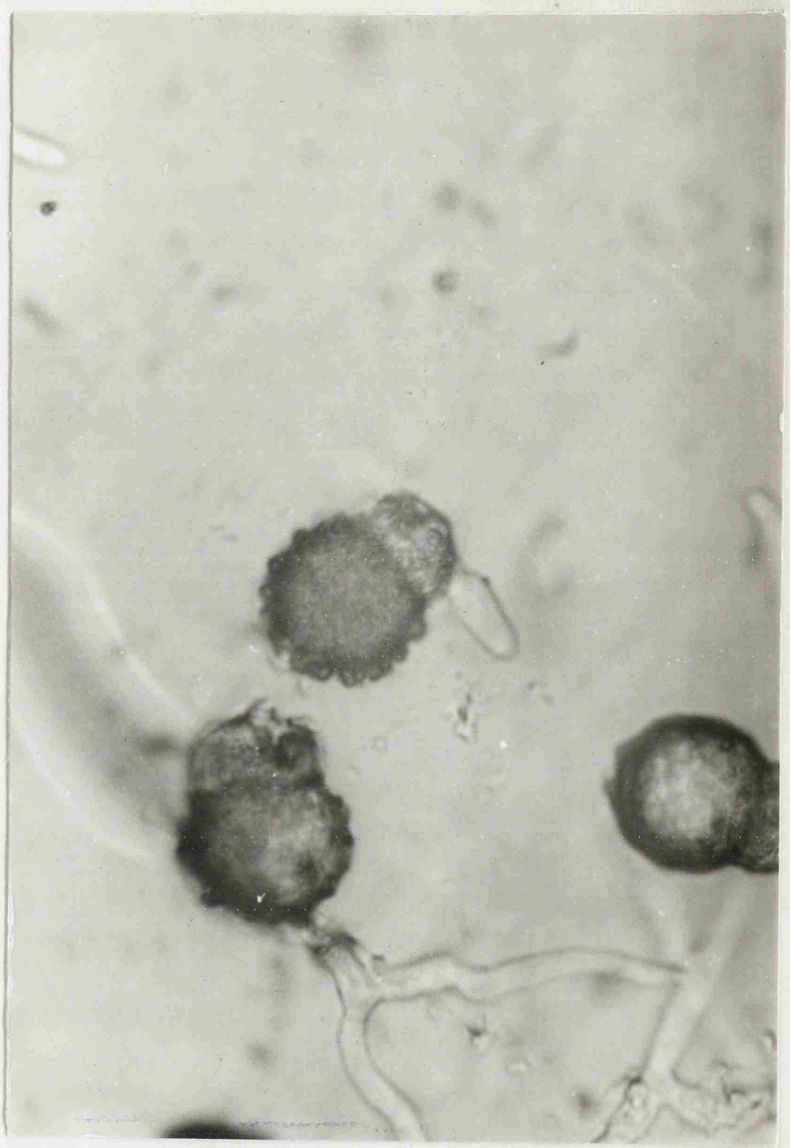
(ii) Experimental

Conidia of V. malthousei and M. perniciosa were found to germinate readily on malt agar after fifteen to twenty four hours incubation at 24 C. However, chlamydospores of M. perniciosa washed from a 14-day malt agar culture failed to germinate on the following agar media :- Corn meal (Oxoid), Corn steep (Oxoid), Czapek Dox (Oxoid), Potato dextrose (Oxoid), Oatmeal, Plain (Oxoid), Malt extract (Oxoid), Malt extract without peptone (Oxoid), a series of malt extract agar plates buffered with phosphate to pH 5.4, 5.8, 6.2, 6.6, 7.0, 7.4 and 7.8, and mushroom extract media A, B and C.* However, where the thin-walled suspending cell was undamaged, germination by a germ tube produced usually at the point of attachment to parent mycelium was consistently observed (Plate 21). Thus the observation by Smith (1924) that chlamydospores from cultures of M. perniciosa germinate readily on mushroom media was not confirmed. This may be because the exact conditions for such germination were not provided or that Smith may have confused germination of the thick-walled cell with that of the basal cell. It is perhaps significant that Smith (1924) illustrated the chlamydospore germinating by producing a germ tube through the basal cell.

The effect of mushroom mycelium on chlamydospores was next examined. Spawn-run compost was filled to the surface of one half

*For composition see Appendix 1.

Plate 21 Germination of the basal cell of a chlamydospore of *M. perniciosa* following incubation on malt agar for 36 hours at 22 C (x 700)



of a rectangular plastic sandwich box, soil being added to the remainder. The box was fitted with a lid and incubated in the growth room to allow mushroom mycelium to fully colonise the soil. A suspension of chlamydospores and conidia was prepared from a 14-day malt extract agar culture of M. perniciosa and sprayed on glass slides which were inverted and placed on the soil in contact with mushroom mycelium. Two such boxes were prepared and each provided with three slides. One slide was removed from each box and examined for spore germination after one, two and three days incubation on the colonised soil at 24 C. Germination of undamaged basal cells of chlamydospores and conidia was repeatedly seen but on no occasion was germination of the thick-walled cell of the chlamydospore seen.

The behaviour of V. malthousei conidia in casing materials was examined with a washed spore suspension sprayed on washed, sterilised slides buried horizontally, spore side uppermost in fresh soil or pasteurized peat adjusted to approximately 50% of maximum water-holding capacities. Other slides were buried in sterilised soil or peat. Slides were stained after one, three and seven days with 0.01% cotton blue in lactophenol following careful removal of excess casing. The percentage germination was determined for each casing type and for control experiments where spores were incubated in water on glass slides or on plain water agar (Table 10). In natural soil, conidial germination of V. malthousei was low and accompanied by rapid lysis of germ tubes, while in sterile soil, high germination

Table 10

Germination of *V. malthousei* conidia on glass slides in casing materials

	Soil		Peat	
	Natural	Sterile	Pasteurized	Sterile
24 hours incubation				
No. germinated spores	41	231	0	201
No. ungerminated spores	183	26	273	12
% germination	18.4	89.9	0	94.4
Comments	Short germ tubes	Long germ tubes		Long germ tubes
48 hours incubation				
No. germinated spores	42	Extensive	4	Extensive
No. ungerminated spores	176	mycelium	296	mycelium
% germination	19.3	and	1.4	and
Comments	Germ tube lysis seen	sporulation observed		sporulation observed
72 hours incubation				
No. germinated spores	8	Extensive	0	Extensive
No. ungerminated spores	192	mycelium	213	mycelium
% germination	4.0	and	0	and
Comments	Germ tube lysis seen	sporulation observed		sporulation observed
Controls	Plain agar		Water	
No. germinated spores	297		69	
No. ungerminated spores	3		194	
% germination	99.0		26.2	
Comments	Long germ tubes		Short germ tubes	

rates and extensive mycelial growth and sporulation occurred [illegible] suggesting the operation of soil fungistasis (Dobbs and Hinson 1953). These observations were even more marked in peat but in natural soil, replacing slides with cellulose acetate membranes, germination was reduced to 45% only, after twenty four hours (Table 11).

Table 11 Germination of V. malthousei conidia on filter membranes in soil.

Spore Treatment	No. spores germinated	No. spores not germinated	Percentage germination
Malt extract agar	208	3	98.5
Water on glass slide	61	143	29.9
Membrane on malt agar	221	0	100.0
Membrane in soil	94	115	45.0

In view of results reported earlier on the survival of V. malthousei in casing, the effect of mushroom mycelium, growing through casing materials, on conidia was next investigated. Spawn-run compost was filled to a depth of 6 cm. in one half of a plastic box 25 x 10 cm and moist soil was filled into the other half and the surface levelled. Two boxes prepared in this way were equipped with close-fitting lids and incubated for twenty five days at 15 - 18 C by which time mushroom mycelium had colonised half the soil 'casing'. Three glass

microscope slides were sprayed with washed spores of V. malthousei, inverted and placed on the soil 'casing' of each box so that one end of each slide was in contact with mushroom mycelium and the other end over uncolonised soil (Fig. 5). The slides were gently pressed down to ensure good contact with the soil, care being taken to avoid damaging mushroom mycelium. One slide was removed from each box, stained and examined after one, three and five days. Spore germination was determined in three distinct zones on the slides - spores in contact with soil only and those in the areas of mushroom mycelium. Within these zones, spore germination percentage were determined in random fields of view. In the last zone, spore counts were made adjacent to individual mushroom hyphae or strands

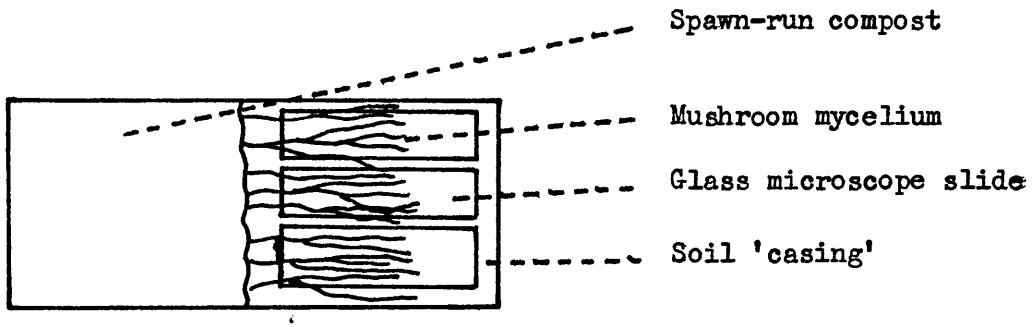


Fig. 5 Plan diagram of plastic box with spawn-run compost and soil 'casing'.

only, in areas where good germination had occurred. The results listed in Table 12 indicate that exudates of mushroom mycelium stimulate spore germination in V. malthousei and can overcome the fungistatic effect observed in soil. These observations were confirmed in several similar experiments. Where germination of conidia had taken place near mushroom mycelium attached to the slide, many of the germ tubes and hyphae of V. malthousei grew towards and alongside mushroom mycelium (Plate 22). After three days conidiophores with verticillate phialides were seen on slides with mushroom mycelium growth, becoming extensive in five days (Plate 23) This effect, normally only seen in sterilised soil is presumably due to utilisation of nutrients exuded from mushroom mycelium since no penetration of host cells by the pathogen was seen. Such exudates however, have no effect on the chlamydospores of M. perniciosa which remain ungerminated when substituted for V. malthousei in similar experiments. Bacterial growth is also supported by mushroom cell exudates (Plate 24).

The factors influencing spore germination were studied further and for convenience, this work is reported in a separate section.

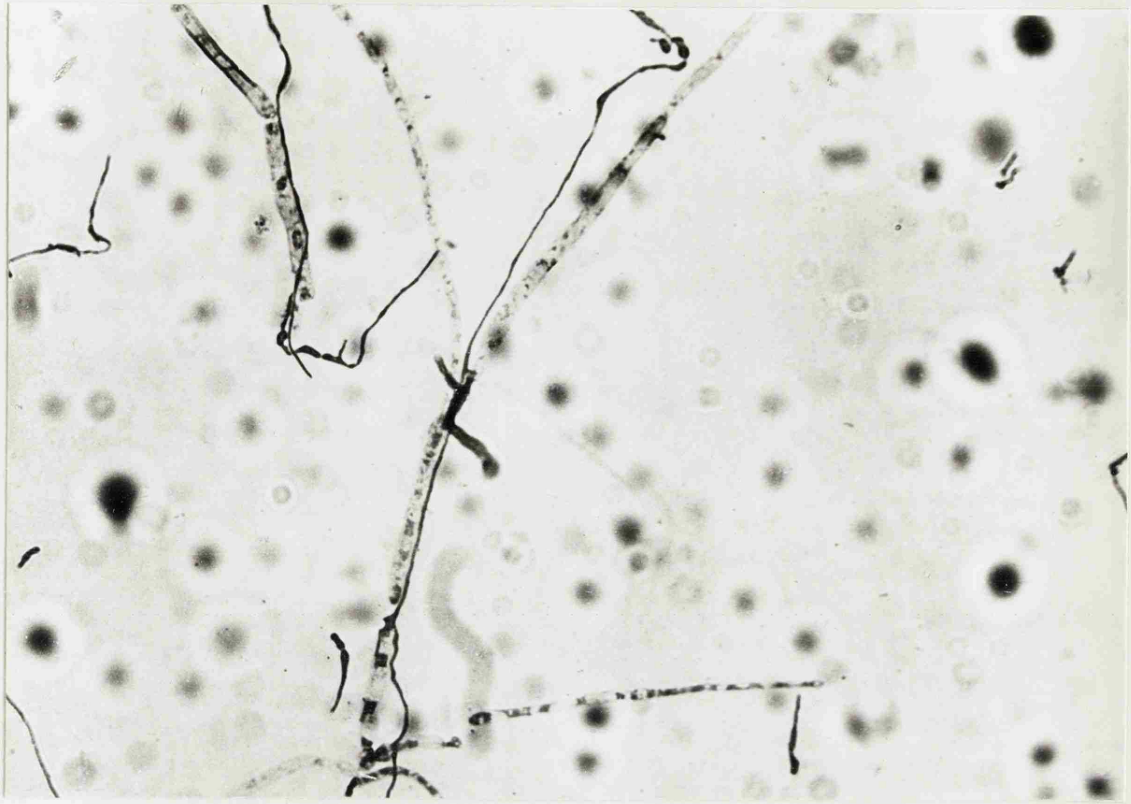
c. Growth of Mycelium in Casing

Experiments to determine the competitive saprophytic ability (Garrett 1956) of V. malthousei were limited to an examination of the colonization of mushroom tissue from soil, and growth into soil from living and dead mushroom tissue. M. perniciosa and D. dendroides

Table 12 Germination of *V. malthousei* conidia in soil on glass slides in contact with mushroom mycelium

Area of slide examined	Incubation period	No. germinated spores	No. ungerminated spores	Percentage germination	Mean % germination
Soil	24 hrs	30	175	14.6	23.5
"	"	66	138	32.4	
Mycelium	"	150	157	48.9	36.7
"	"	60	185	24.5	
Selected areas adjacent to mushroom mycelium	"	202	41	83.1	82.9
	"	171	36	82.6	
Soil	72 hrs	12	228	5.0	15.1
"	"	54	160	25.2	
Mycelium	"	162	224	42.0	37.5
"	"	76	156	32.8	
Selected areas adjacent to mushroom mycelium	"	193	18	91.5	85.6
	"	168	43	79.6	

Plate No. 22 Germination of V. malthousei conidia on a glass slide in soil near mushroom mycelium. Growth of germ tubes alongside mushroom mycelium adhering to the glass slide is seen. (X1000)



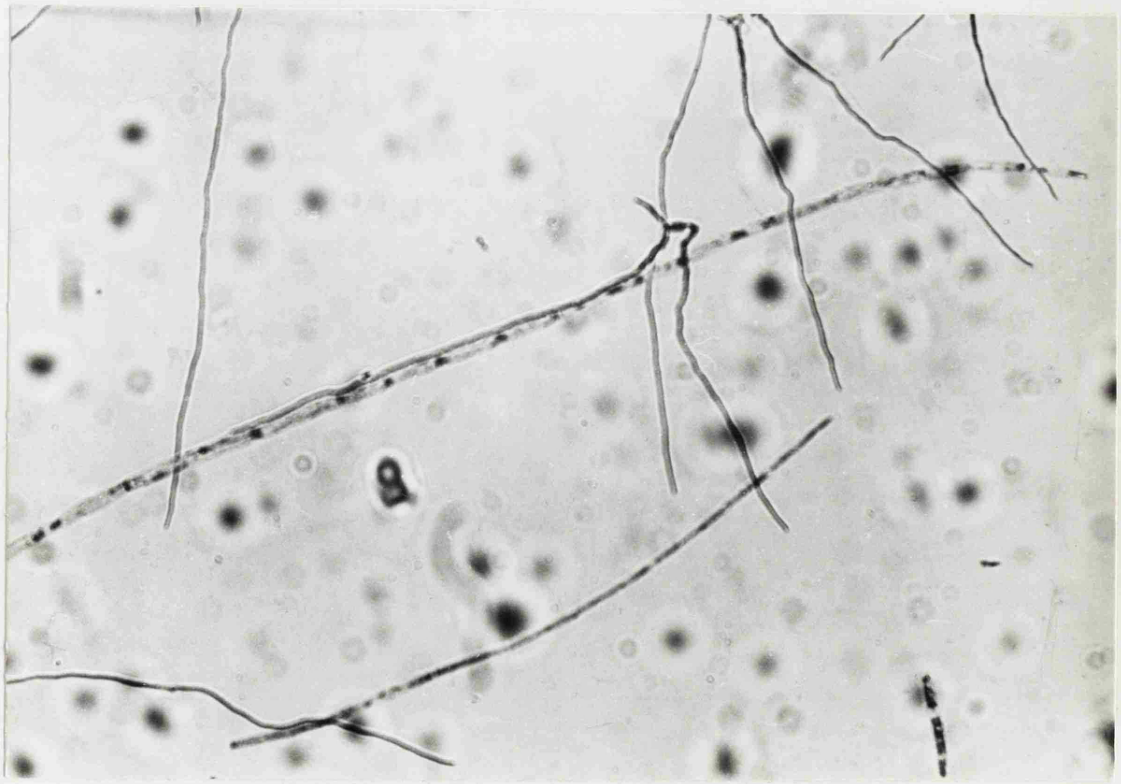


Plate No. 23 Verticillate conidiophores seen on glass slide coated with conidia of *V. malthousei*, placed on soil in contact with mushroom mycelium (x 700)

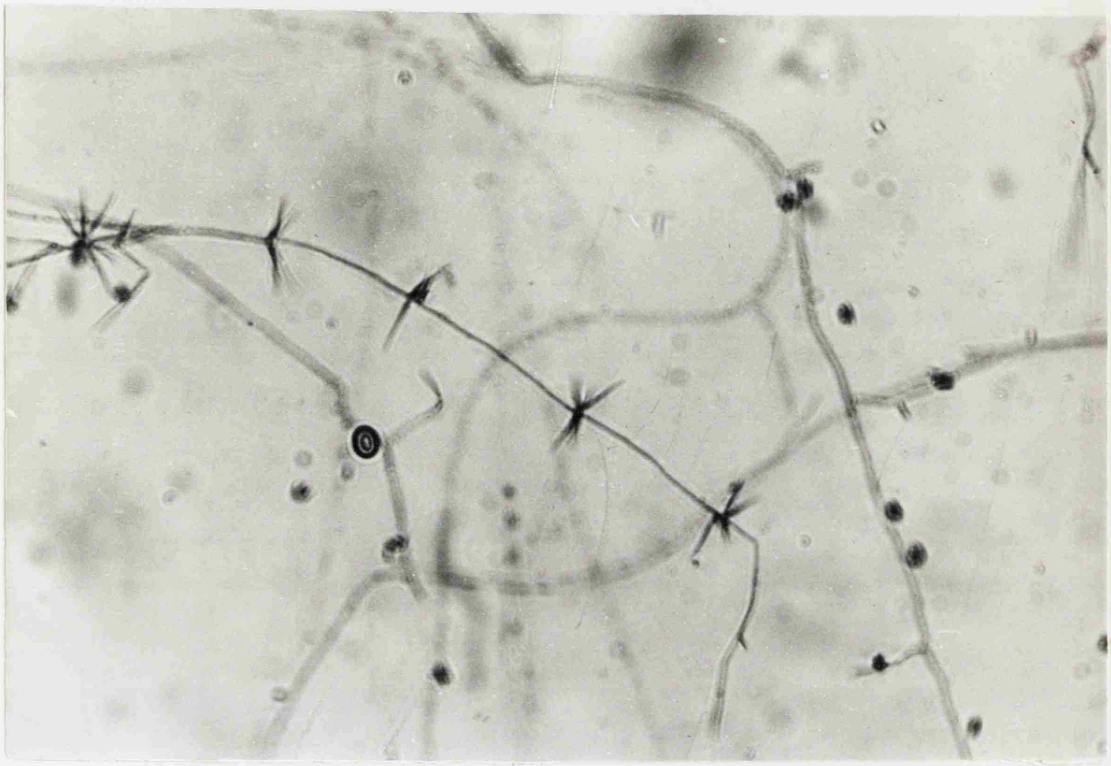
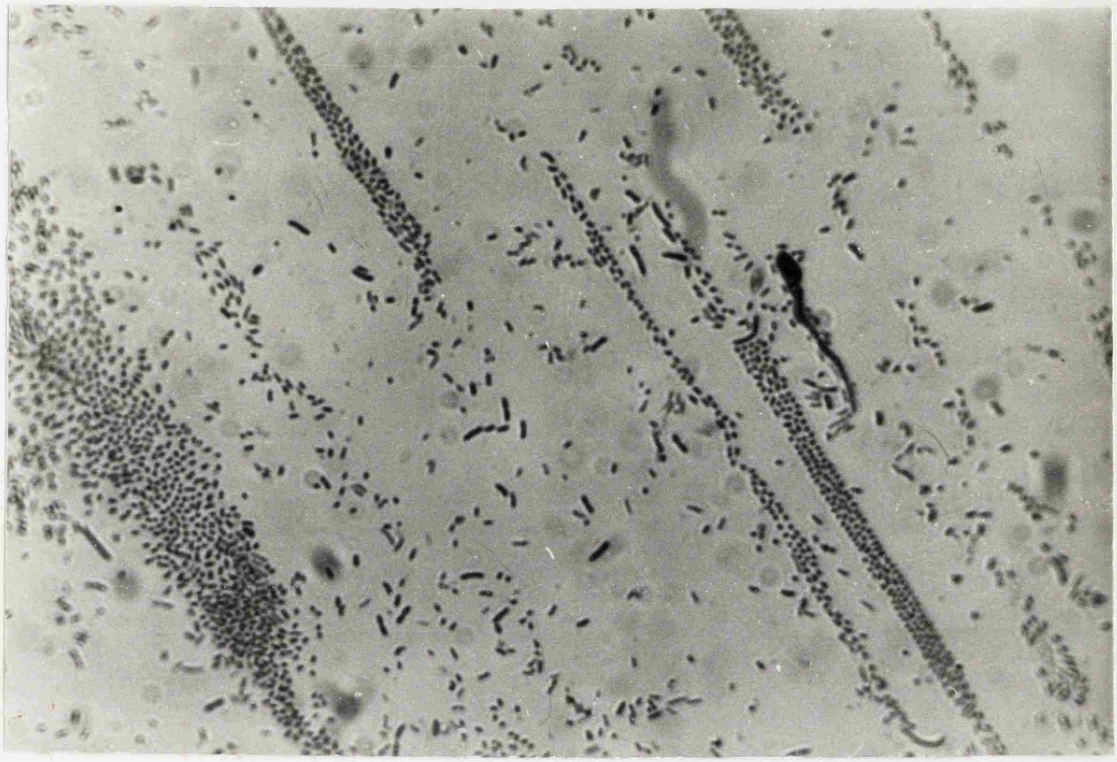


Plate No. 24 Bacteria observed on glass slides placed in contact with mushroom mycelium. A germinating spore of V. malthousei is visible. (X 2000)



were included in one experiment.

The colonization of sterile and non-sterile mushroom stalk tissue by V. malthousei was examined in sterile and non-sterile soil (50% of maximum waterholding capacity) respectively. The stalks were dipped in a suspension of conidia (approximately 2.6×10^8 per ml) before incoporation into the soil and after incubation at 24 C for seven days the soil surfaces were examined microscopically for sporulation of the pathogen. Soil was then carefully removed to expose the stalks which were also examined for surface growth of the pathogen. After transfer to sterile petri-dishes, the stalks were broken open and pieces of tissue from the centre aseptically transferred to malt agar plates. Soil particles adjacent to the stipes (up to 5 mm.) and from the area 5 - 10 mm. from the stipes was suspended in sterile water. Serial tenfold dilutions were made of each soil suspension and 1 ml. of each dilution was mixed with molten, cooled malt extract agar in a petri-dish. The plates were incubated for several days at 24 C and were examined daily for the presence of V. malthousei colonies (Table 13). Thus mushroom tissue in unsterilised soil can be colonized by V. malthousei when high inocula are employed. Also the inoculum potential of the pathogen in soil is increased by sporulation on the buried stipes. However, mycelial growth from the food base was not detected in competition with the microflora of natural soil as no colonies of V. malthousei were isolated from soil 5 - 10 mm from the stipe although colonies were readily isolated from soil immediately adjacent

Table 13

Sporulation and isolation of *V. malthousei* from colonized mushroom stipes and from soil

Treatment		Observed sporulation		Cultures of *V. malthousei* from :-		
Soil	Stipe	Soil Surface	Stipe surface	Stipe centre	Soil 0-5mm from stipe	Soil 5-10mm from stipe
Sterilised	Sterilised	+	+	+	+	+
"	"	+	+	+	+	+
Natural	Unsterilised	-	+	+	+	-
"	"	-	+	+	+	-

Key + Sporulation or colony present

\- Sporulation or colony absent

to the stipe. Thus it is concluded that V. malthousei is poorly equipped to compete with the soil microflora and grow from a nutrient source into soil.

These conclusions were substantiated using a modification of the recolonization tube technique of Evans (1955) involving the use of 6 cm. lengths of 5 mm. bore P.V.C. tubing, which was cut into 5 mm. segments for isolation purposes (Fig. 6). M. perniciosa and D. dendroides were also included in this experiment. After introduction of infected mushroom tissue at one end of tubes containing sterilised or unsterilised soil and incubation for seven days at 24 C the 5 mm. rings cut from two

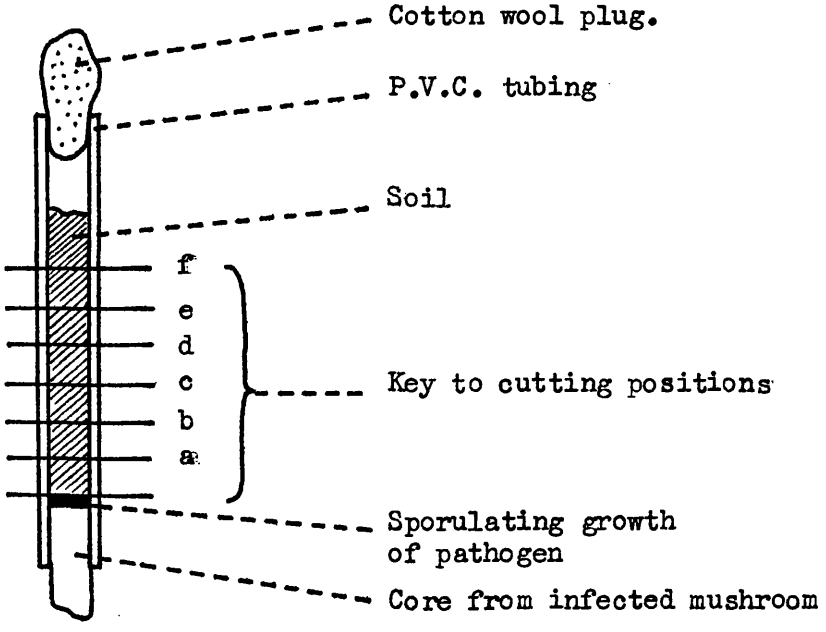


Fig. 6. Diagram of inoculated soil tube showing key to cutting positions.

tubes of each treatment were each aseptically placed distal end downwards on a clean fresh cut button mushroom and covered by a plastic beaker. Soil from rings cut from other tubes was distributed over malt extract agar containing 1 : 30,000 rose bengal and 30 µg/ml streptomycin. After five days incubation at 15 - 18 C in the dark the plates and mushrooms were assessed (Tables 14, 15 and 16). The mean water content of soil used was 25.0% of dry weight or 38.5% of maximum waterholding capacity.

Table 14 Growth of *V. malthousei* on duplicate plates and mushrooms inoculated with soil from growth tubes

Tube Section (see Fig. 6)	Sterile Soil		Non sterile Soil	
	Plate	Mushroom	Plate	Mushroom
a	+ +	+ +	+ +	+ +
b	+ +	+ +	- +	- -
c	+ +	+ +	- -	+ +
d	+ +	+ +	- -	+ -
e	- +	- +	- -	- -
f	- -	- -	- -	- -

Key to Table 14.

\+ Growth of *V. malthousei* observed

\- Growth of *V. malthousei* not observed

Plate = Plate detection method

Mushroom = Mushroom detection method

Table 15 Growth of *M. perniciosa* on duplicate plates and mushrooms inoculated with soil from growth tubes

Tube Section (see Fig. 6)	Sterile Soil		Non sterile Soil	
	Plate	Mushroom	Plate	Mushroom
a	- -	+ +	- -	- -
b	- -	- -	- -	- -
c	- -	- -	- -	- -
d	- -	- -	- -	- -
e	- -	- -	- -	- -
f	- -	- -	- -	- -

Table 16 Growth of *D. dendroides* on duplicate plates and mushrooms inoculated with soil from growth tubes

Tube Section (see Fig. 6)	Sterile Soil		Non sterile Soil	
	Plate	Mushroom	Plate	Mushroom
a	+ +	+ +	+ +	- -
b	- -	+ +	- -	- -
c	- -	+ +	- -	- -
d	- -	- -	- -	- -
e	- -	- -	- -	- -
f	- -	- -	- -	- -

Key to Tables 14, 15 and 16.

\+ Growth of *V. malthousei* observed

\- Growth of *V. malthousei* not observed

Plate = Plate detection method

Mushroom = Mushroom detection method.

The plate isolation method indicated for non-sterile soil that the first 5 - 10 mm. of soil were colonized by the pathogen. The mushroom detection method, used as an attempt at a selective isolation procedure, gave more variable results probably due to natural infection by the pathogen inferred from the infection of one mushroom out of ten covered with uninoculated soil. However, the results of this and previous experiment suggest that V. malthousei is characterised by low competitive saprophytic ability. Although M. perniciosa on mushroom beds exhibits limited colonization of the casing surface from infected sporophores while D. dendroides is able to extensively colonize the casing surface, the results indicate they are not able to grow below the surface of the casing from infected mushrooms.

d. Infection

The infection of mushroom sporophores by V. malthousei was investigated using various inoculation techniques and spore concentrations.

To determine the most effective method to infect mushroom sporophores with V. malthousei, spawn-run compost in fibre pots was inoculated in three ways - 2.5 ml. spore suspension from a 7-day malt extract agar culture was sprayed on the compost surface or mixed in the peat casing or sprayed on the casing surface of each pot. Each treatment was prepared for twenty pots and the inoculation for each pot was approximately 4.3×10^7 conidia.

Casing materials consisted of 50g. peat with limestone chippings in each replicate. Twenty uninoculated controls were prepared. Other controls consisting of five pots per treatment were set up using water instead of a spore suspension. Numbers of healthy and diseased mushrooms from one flush in each treatment were recorded (Table 17).

Table 17 Numbers of diseased and healthy mushrooms produced in an infection experiment with *V. malthousei* conidia

Treatment	No.mushrooms infected	No.mushrooms healthy	% mushrooms infected
Conidia on compost surface	7	56	11
Conidia within casing	77	4	95
Conidia on casing surface	42	26	62
Uninoculated control	-	47	-
Water on compost surface	-	11	-
Water within casing	-	11	-
Water on casing surface	-	10	-

Although some infection was produced by compost inoculation much greater rates of infection were obtained from inoculated casing, particularly when the conidia were mixed throughout the casing. This indicates that typical sclerodermoid symptoms result from an attack on strands or sporophores within or at the surface of the casing rather than on mushroom mycelium on the compost.

To investigate the effect of inoculum potential of V. malthousei in casing materials, an infectivity assay was carried out by mixing conidia from 7-day cultures with peat with limestone or soil casing materials made up to 50% of maximum waterholding capacity, the spore concentrations used being recorded with the results in Table 18 and Fig. 7. The concentration of spores required to produce infected mushrooms in 50% of pots was higher for soil than for peat.

Table 18 Number of pots producing infected mushrooms in infectivity assay of conidia of V. malthousei

Peat Casing			Soil Casing		
Number spores/g.	No. pots with infection	No. pots without infection	Number spores/g.	No. pots with infection	No. pots without infection
0	-	10	0	-	10
1.45×10^2	-	10	81	-	10
1.45×10^3	2	8	8.1×10^2	-	10
1.45×10^4	8	2	8.1×10^3	1	9
1.45×10^5	10	-	8.1×10^4	10	-

The behaviour of V. malthousei immediately prior to infection of the mushroom was examined more closely using a deep casing technique. Tall 1 L glass beakers were half-filled with spawn-run compost. A washed spore suspension of V. malthousei was mixed with peat and limestone chippings and half of this mixture was used to

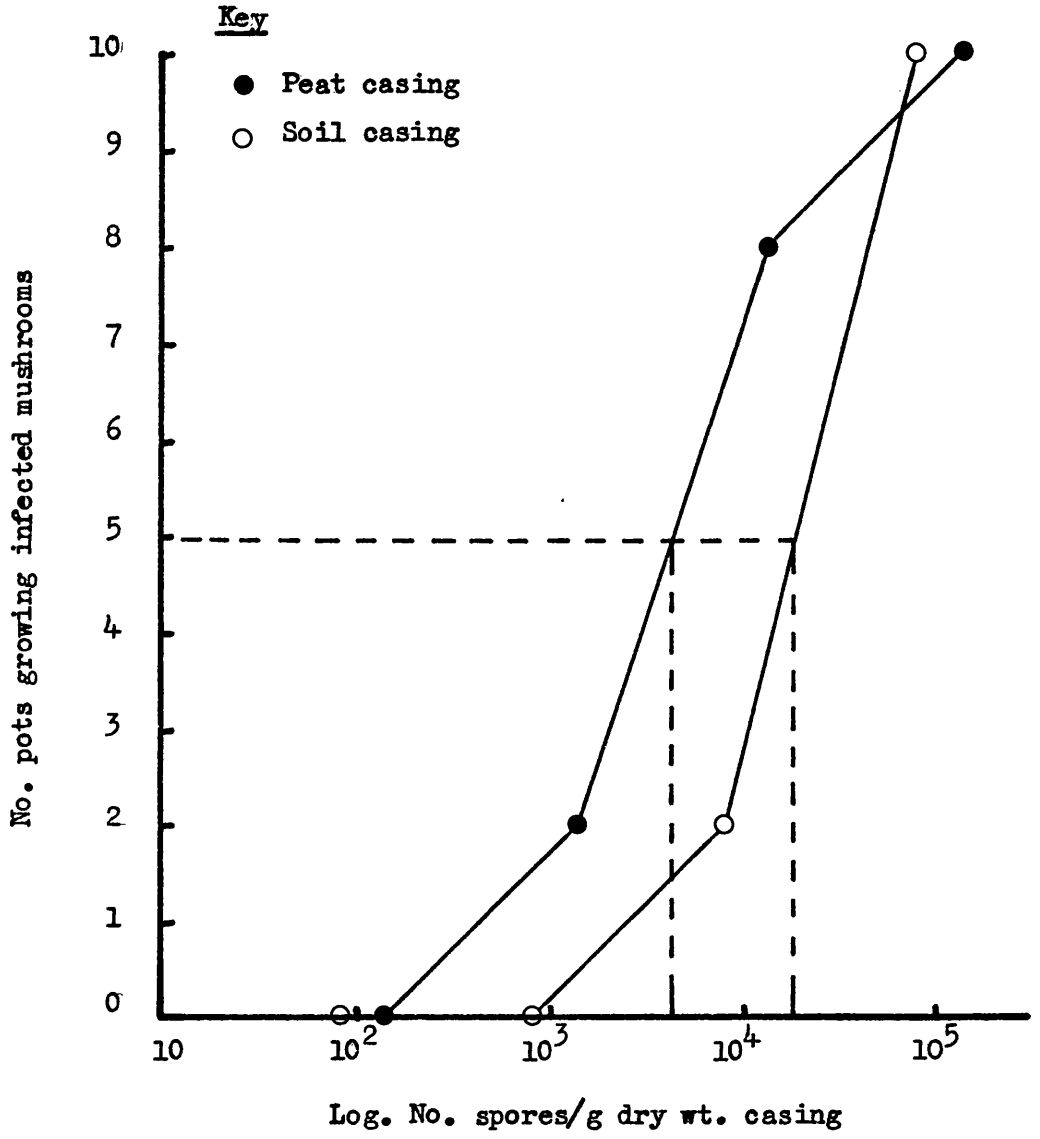


Fig.7 No. pots growing infected mushroom sporophores in infectivity assay with V. malthousei

provide a casing layer 1 cm. deep over the spawn-run compost in three beakers. Care was taken to avoid contaminating the sides of the beakers above this level. Additional moist peat was placed on top of this layer until the casing was 8 cm. deep. Three more beakers were cased with 7 cm. moist peat and overlaid with a 1 cm. depth of inoculated peat. Two beakers with casings of uninoculated moist peat 8 cm. deep served as controls. Polythene sleeves were secured with rubber bands to the sides of the beakers just below the rims and to rubber bungs clamped 10 cm. above to enclose the casing allowing surface carbon dioxide to accumulate initially, preventing sporophore initiation and encouraging vegetative growth (Long and Jacobs 1969). The bung admitted a short air inlet tube and an air outlet tube which extended to within 5 mm. of the casing. A Charles Austin oil-free diaphragm pump supplied air under positive pressure through a flow-meter, branched supply pipes and humidification flasks. All beakers were incubated for four weeks at 15 - 18 C during which no fresh air was supplied. When the mushroom mycelium had grown to within 5 mm. of the casing surface, air was supplied at a rate of 2 L/min per beaker to promote sporophore initiation which normally occurred in the top 5 mm. of casing.

The three beakers inoculated in the upper layer of casing produced only sclerodermoid infected mushrooms. Normal shaped mushrooms bearing extensive sporulation of the pathogen on the pileus, gills and stipe and sclerodermoid mushrooms were formed in beakers

inoculated at the bottom of the casing. Portions of casing abutting the side of beakers of all treatments were carefully removed intact and were examined microscopically. All strands from all treatments appeared normal and healthy, even those below infected initials (Plates 25 and 26). However, the tissues of developing initials were covered with sporulating heads of V. malthousei irrespective of their position within the casing layer. Several instances were noted in beakers inoculated at the bottom of the casing of a mass of infected sporophore tissue formed deep in the casing covered by sporulation of the pathogen yet linked by apparently healthy strands to other diseased sporophores in the surface layers of casing (Plate 27).

The occurrence of V. malthousei on diseased sporophores at the casing surface in pots inoculated at the base of the casing indicates that spores of the pathogen had germinated and grown through the peat to the surface. From previous results showing the relationship between parasite and mushroom mycelium this growth is probably closely associated with mushroom mycelium. In a repeat of this experiment an attempt was made to detect V. malthousei in mushroom strands or in peat near strands from the upper 4 cm. of casing inoculated basally. Sections of peat abutting the sides of the beakers were again removed intact, care being taken to avoid contamination of one layer of casing by another. Ten pieces of mycelium strand were dissected out aseptically and transferred to the surface of clean button mushrooms covered by plastic beakers.

Plate 25 Mushroom strand in casing with infected sporrophore at top covered by sporulation of V. malthousei. Montaged prints rephotographed (X 25)

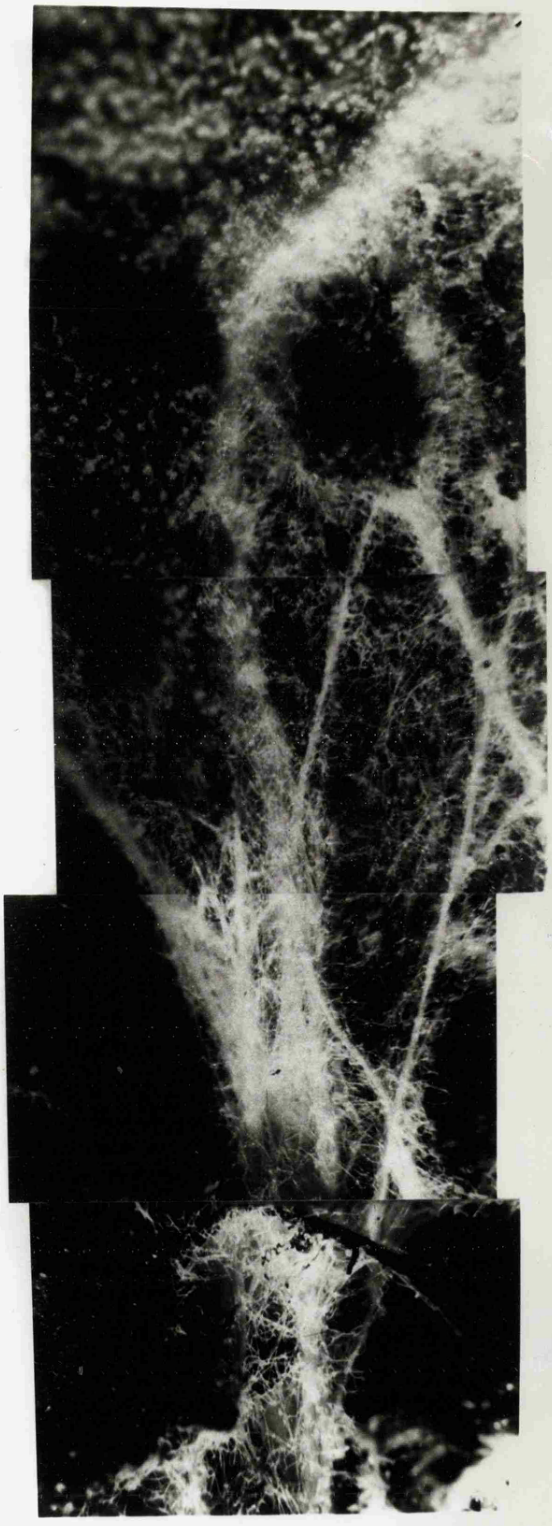


Plate 26 Healthy mushroom strands in casing. Montaged prints rephotographed. (X 25)

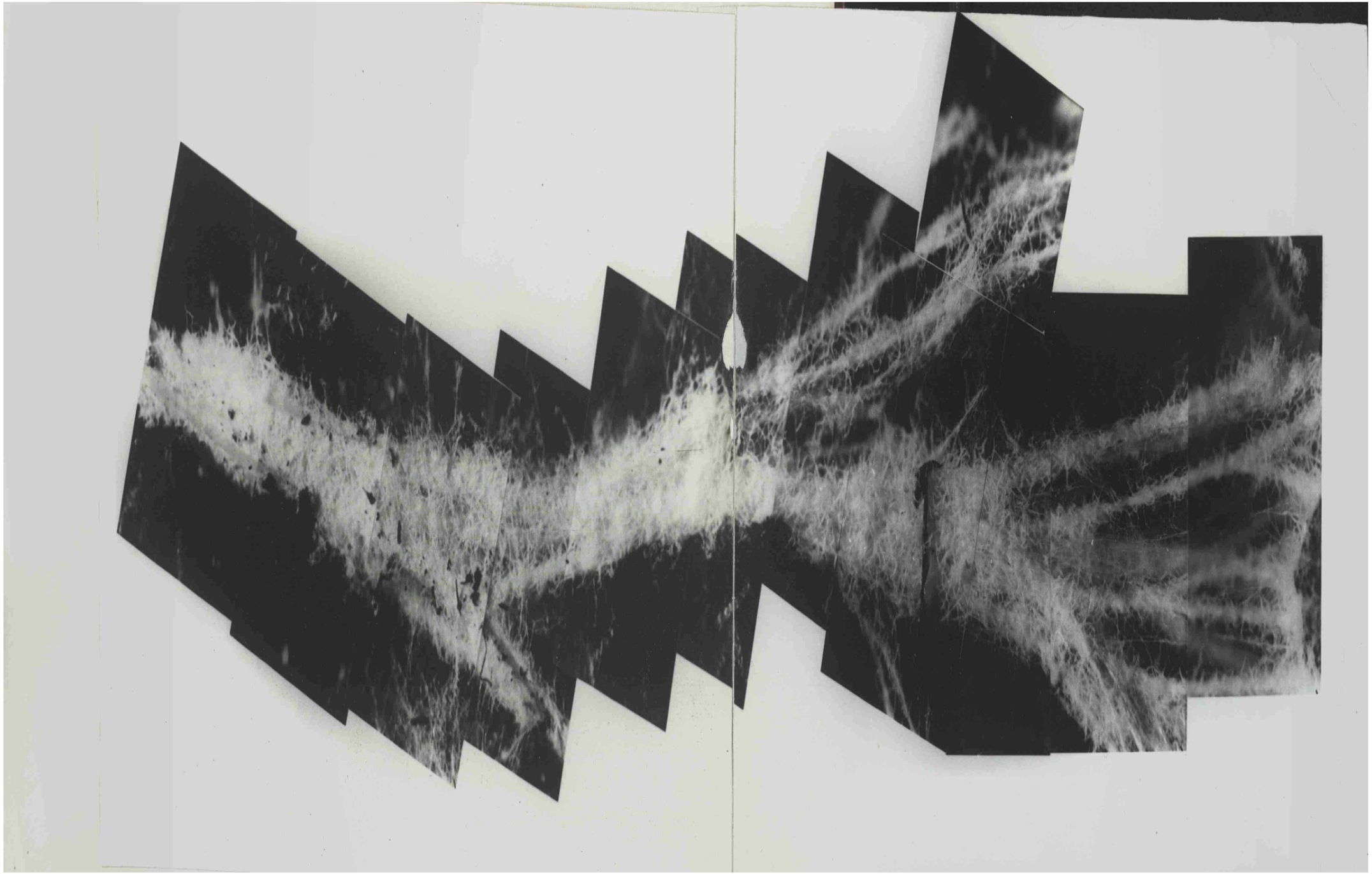
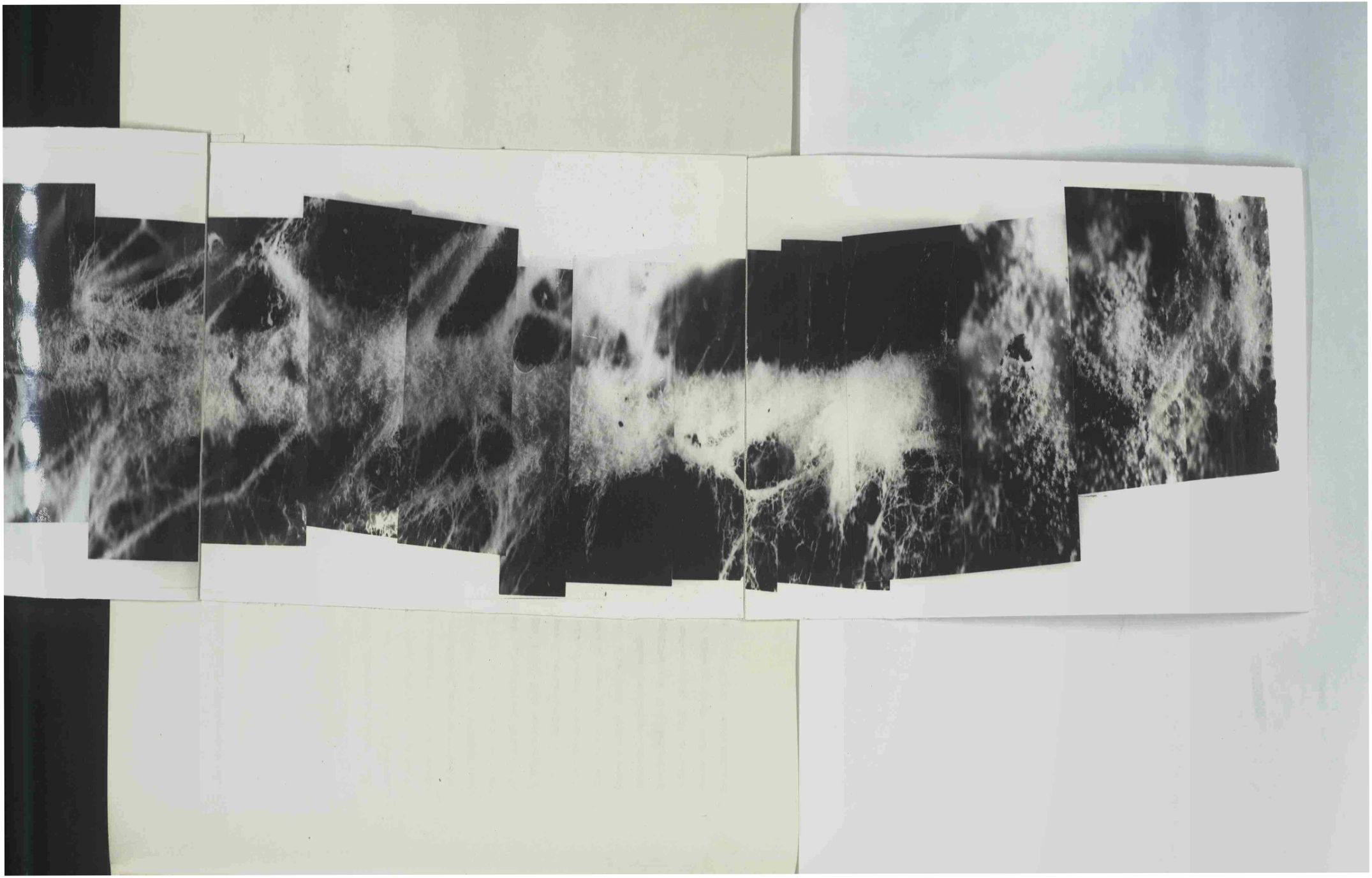


Plate 27 Apparently healthy strand linking mushroom initial at top with mass of sporophore tissue formed deep in casing, both infected with V. malthousei and covered with sporulation of the pathogen. Montaged prints rephotographed (x 25)



A further ten strands were similarly removed from inoculated and control treatments and placed on malt extract agar in petri-dishes. Peat particles adjacent to these strands were plated out on ten malt extract agar plates per treatment. After incubation for several days at 24 C, mushrooms and agar plates were examined for V. malthousei.

The results listed in Table 19 show that few isolations of V. malthousei from strands or peat were made possibly because the mycelium of the pathogen is difficult to isolate and detect or that it may not be exclusively associated with mushroom mycelium. Several strands from each treatment were then removed from the casing as before, fixed in formalin acetic alcohol, dehydrated, embedded in 'Paraplast' wax, sectioned on a Reichert rotary microtome to 15 µ thickness and stained using the periodic acid Schiff method, but mycelium of V. malthousei was not detected. (Plates 28 and 29).

Disease symptoms observed with deep casing experiments indicate that the most severe infections resulted from inoculum in the upper layers of casing. The less severe infections in basally inoculated casing are concluded to result from conidial germination and growth of V. malthousei through to the surface layers of casing. Failure to detect pathogenic mycelium in sections of strands connected to infected sporophores is probably due to the relatively few hyphae of V. malthousei present in the mushroom strand and the lack of a selective staining method. The

Table 19

Isolation of *V. malthousei* from mushroom strands and peat from casing using the malt agar plate and mushroom cap isolation methods

Basally Inoculated Casing			Uninoculated casing			Controls	
Mushroom isolation	Malt agar isolation		Mushroom isolation	Malt agar isolation		Uninoculated mushrooms	Inoculated mushrooms
Strands	Strands	Peat	Strands	Strands	Peat		
-	-	+	-	-	-	-	+
-	-	-	-	-	-	-	+
-	-	-	-	-	-	+	+
-	-	-	-	-	-	-	+
-	-	-	-	-	-	-	+
-	-	-	+	-	-	-	+
-	+	-	-	-	-	-	+
-	-	-	+	-	-	-	+
-	-	+	-	-	-	-	+
-	-	+	-	-	-	-	+

Key :- *V. malthousei* spore-heads observed (+) or not observed (-)

structural similarity of strands connected with infected and healthy mushroom sporophores indicates that V. malthousei causes no apparent changes or symptoms in the strands but attacks the sporophore of the cultivated mushroom.

Plate 28 Section of mushroom strand linked with healthy sporophore initial in uninoculated casing. (X 1000)

Plate 29 Section of mushroom strand linked with sporophore initial infected with *V. malthousei*. (X 1000)

6. THE NATURE OF THE INHIBITION OF GERMINATION OF V. MALTHOUSEI CONIDIA IN CASING MATERIALS.

Previous results in the present study indicated that V. malthousei conidia in soil exhibit behaviour which can be interpreted as soil fungistasis (Dobbs and Hinson 1953). Among the first workers to recognise the inhibition of germination of fungal spores in soil were Simmonds et al (1950) with Helminthosporium sativum, Hessayon (1953) with Trichoderma Viride and Chinn (1953) who recorded the failure of several species to germinate in soil. Later that year Dobbs and Henson extended these observations and framed a general concept as a 'widespread fungistasis in soils'. Since this report by Dobbs and Henson (1953) the concept of soil fungistasis or soil mycostasis (Dobbs and Bywater 1957) has been verified by numerous workers using different fungi, soils and techniques in several countries (Lockwood 1964).

The mechanism of soil fungistasis is the subject of current debate. Most workers in this field subscribe to the view of Dobbs and Hinson (1953) that the inhibition is caused by a widespread diffusible fungistatic factor of microbial origin which may be masked by nutrients and removed by severe heating or desiccation. However, the nature of the inhibitor is viewed differently by different workers. Antibiotics (Lockwood 1959), volatile compounds (Schuepp and Green 1964, Robinson and Park 1966, Balis and Kouyeas 1968, Robinson, Park and Garrett 1968), lignin breakdown products

(Lingappa and Lockwood 1962), carbon dioxide (Brian 1960, Bourret, Gould and Snyder 1968), culture staling products (Park 1961) and antimetabolites of glucose (Dobbs 1962) have all been considered but despite numerous attempts these have never been satisfactorily extracted - sterile aqueous extracts rarely inhibiting spore germination and sometimes being stimulatory (Dobbs 1962, Bumbieris and Lloyd 1967, Schuepp and Green 1968, Vaartaja and Agnihotri 1968, Willis and Williams 1968). It has been pointed out that the inhibitor must be resistant to leaching and to chemical and biological inactivation since it persists in fallow soil for several years (Jackson 1958).

Another hypothesis for the mechanism of soil fungistasis was mentioned as a possibility by Brian (1960) and proposed as an alternative by Lockwood (1964) and depends on microbial competition for nutrients resulting in the unavailability in soil of materials required for spore germination. A presupposition of this hypothesis is that most fungal spores fail to germinate in distilled water in the absence of exogenous nutrients, which is refuted by Dobbs and Hinson (1953), Park (1960) and Lingappa and Lockwood (1961). However many of the experiments from which these conclusions were drawn were carried out with no record of procedures to remove contaminating nutrients from spore suspensions or agar assay media, a serious omission since many fungal spores have been shown to require external nutrients for germination (Cochrane 1960, Ko and Lockwood 1967, Menyonga 1967, Mircetich, Zentmeyer and Kendrick 1968, Griffin

and Pass 1969).

a. Spore Germination in Relation to Nutrients

Conidia of Verticillium albo-atrum were shown to be susceptible to soil fungistasis by Powelson and Patil (1963), which was attributed to a fungistatic principle by Schreiber and Green (1963) who also showed that it could be overcome by plant root exudates - susceptible being more effective than non-susceptible plants. Powelson (1966) concluded that the major factor limiting germination of conidia of Verticillium dahliae in soil was the exogenous availability of diffusible carbon substrates. The work reported below examines factors influencing germination of conidia of V. malthousei in soil. Methods used in spore germination experiments were mainly as described earlier (page47) when it was established that conidia of V. malthousei germinate readily on a range of common laboratory agar media, in sterilised soil and in the presence of mushroom mycelium exudate. For the germination of conidia in the absence of exogenous nutrients, twice-washed spores suspended in distilled water were used and germination estimated in water and on plain agar (Table 20). Clearly sufficient nutrients to support a high rate of germination of washed V. malthousei conidia are present in plain agar. Reduced germination levels in water are due either to many of the spores possessing a requirement for exogenous nutrients for germination or to the loss of essential materials from spores during washing.

Table 20 Germination of washed V. malthousei conidia incubated in water or on plain agar.

Percentage Germination of Replicates				
Distilled water		Plain ion-agar		
45.7	55.2	99.0	94.5	96.1
32.0	57.3	85.3	97.1	94.6
27.4	15.5	95.0	87.0	96.5
41.5	32.6	95.0	91.8	86.7
22.9	13.9	60.7	91.5	95.1
18.1	39.5	80.7	94.6	91.1
61.3		80.0	98.7	86.1
		84.1		

To test this latter suggestion, conidia were removed mechanically from a 7-day culture of V. malthousei by carefully touching spore-masses with a nichrome wire. These conidia were suspended in droplets of water at approximately 5×10^5 spores/ml and incubated on glass slides. The results (Table 21) show that germination of such conidia in water is further reduced, indicating that over 90% of V. malthousei conidia have a requirement for external nutrients for germination. Repeated washing of spores (Table 22, Fig. 8) does not reduce germination suggesting that essential nutrients are not removed from conidia which have the capacity to germinate.

The possibility that other nutrients are removed during washing

Table 21 Percentage germination of conidia removed mechanically from cultures of V. malthousei and incubated in water or on malt agar.

Incubation medium	No. germinated spores	No. ungerminated spores	Percentage germination
Water	21	199	9.5
"	17	212	7.4
"	19	208	8.4
Malt agar	202	2	99.0

Table 22 Percentage germination of washed conidia of V. malthousei.

No. washes	Incubation medium	No. germinated spores	No. ungerminated spores	Percentage germination
2	Water	42	83	33.6
4	"	30	70	30.0
6	"	61	133	31.6
8	"	35	91	27.8
10	"	44	87	35.6
12	"	58	124	31.9
2	Malt agar	134	12	91.8
12	"	140	13	91.5

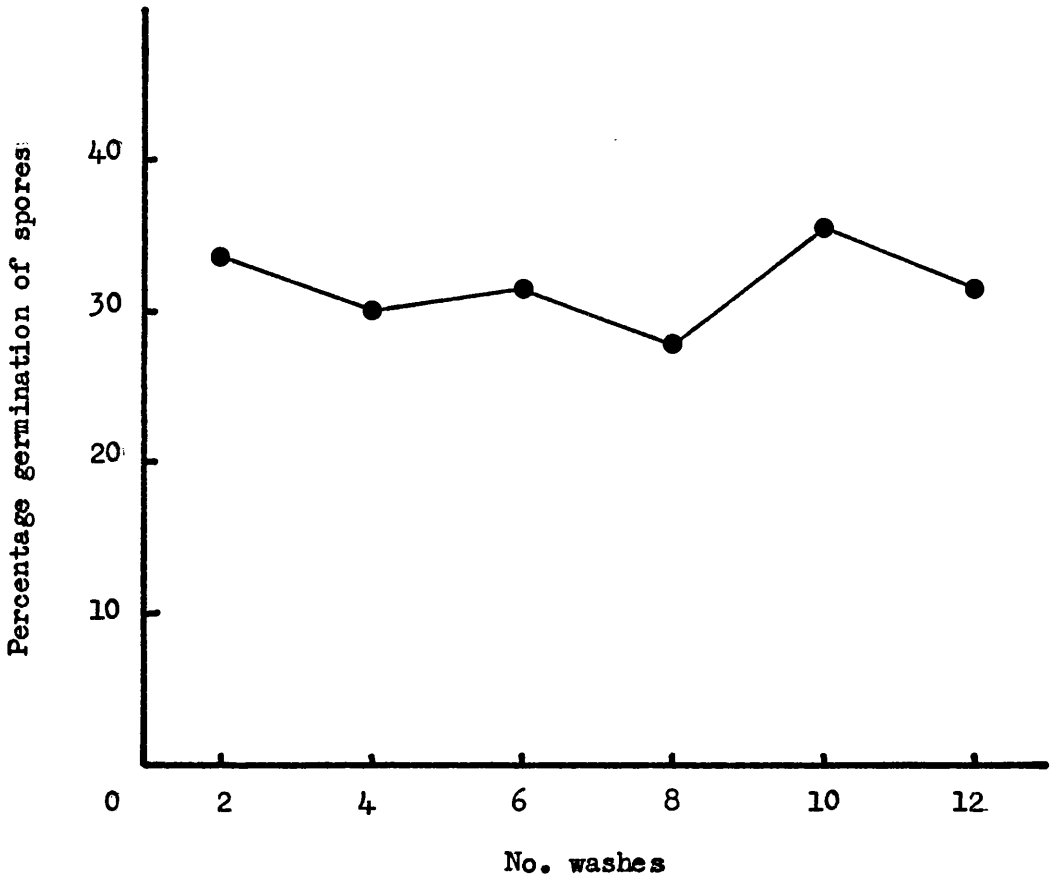


Fig.8 The effect of repeated washing on the germination of conidia of V. malthousei.

was further examined using the approach of Lingappa and Lockwood (1964) who showed for species of Puccinia, Glomerella and Neurospora that nutrients in the exudate from spores in soil stimulated a 3 - 10 fold increase in numbers of soil bacteria. A washed suspension of V. malthousei conidia containing approximately 8.4×10^6 spores/ml was distributed aseptically in 2 ml. aliquots to six sterile boiling tubes containing 5 g. fresh soil (up to 2 mm. particle size and at 38.5% of maximum water-holding capacity) and to six tubes of similar soil sterilised by heating to 121 C for two hours in an autoclave. Initially and after twenty four hours dilution plate counts of bacteria were made in nutrient agar (Table 23). The effect of adding conidia is very similar to that of adding water - the percentage increase in soil bacteria after twenty four hours exposure to conidia or water alone being 64.0 and 68.2 respectively - and although only three replicates per treatment were used the results do not compare with those obtained by Lingappa and Lockwood (1964), suggesting that significant loss of nutrients from washed conidia of V. malthousei in soil does not occur.

b. Spore germination in casing extracts

The effect of water extracts of soil on germination of V. malthousei conidia was examined. Aliquots of 100 ml. distilled water were added to two flasks containing 50 g of 2 mm. sieved, sterilised (121 C for ninety minutes) and non-sterilised fresh soil containing

Table 23

Numbers of bacterial colonies obtained from soil.

Inoculum	Incubation (hr)	Nos. bacteria Assay x 10^4	Mean No./g dry soil
Water	0	50	
"	0	53	133250
"	0	57	
"	24	79	
"	24	105	224175
"	24	89	
Spores	0	88	
"	0	56	169175
"	0	59	
"	24	146	
"	24	80	277500
"	24	107	

25% moisture. Both were shaken and allowed to stand for twenty-four hours at 24 C when extracts were decanted, centrifuged at 4000 rpm. and sterilised by membrane filtration. Another flask of unheated soil was incubated for five minutes only before extraction. A similar series of extracts were prepared from soil dried and stored for several months. One ml. of each extract was

Table 24 Germination of conidia of V. malthousei in soil extracts

Treatment		No. spores germinated	No. spores ungerminated	Percentage germination
Soil	Extraction period			
Fresh Sterile	24 hrs	199	7	96.6
" Non sterile	5 mins	165	53	75.5
" "	24 hrs	190	91	67.6
Dry Sterile	24 hrs	196	6	97.0
" Non sterile	5 mins	162	51	76.0
" "	24 hrs	177	64	73.4
Malt extract agar		206	2	99.0
Water		100	141	41.5

mixed with an equal volume of washed spore suspension of V. malthousei and droplets were incubated on glass slides. Extracts of non-sterile soils by both methods (Table 24) showed an increase in germination (67 - 76%) over distilled water controls (41%) while extracts of sterilised soils showed higher levels of germination due either to destruction of inhibitors or to increased concentrations of nutrients due to autoclaving.

Varying the temperature of the twenty four hour extraction period (Table 25) demonstrated that germination was promoted except at 24 C but the results at this temperature are variable (see Table 24). Failure to stimulate germination by extraction of

Table 25 Germination of conidia of V. malthousei in soil extracts.

Extraction Temperature (C.)	No. germinated spores	No. ungerminated spores	Percentage germination
1	198	33	85.7
11	165	40	80.5
24	58	150	27.9
45	171	57	75.0
Conidia in water	60	160	27.4

soil maintained at 24 C could be related to microbial activity at this temperature compared with other treatments. Such activity could deplete soil and the water extract of soluble nutrients or

result in the production of materials which directly inhibit germination. The similarity between the results for the 24 C extract and the distilled water control suggests that the former conidition may be operative. High germination values of extracts in the previous experiment could be due to increased nutrients or reduced fungistatic materials extracted.

The effect of a mushroom sporophore exudate and heat treatment on water extracts of unsterilised soil and peat was next examined. Water extracts of natural and sterilised soil and peat casing material were prepared as before at 24 C and sterilised by membrane filtration. Part of these extracts of non-sterile casing were then heated at 121 C for one hour in an autoclave. A mushroom exudate solution prepared by placing 30 g young button sporophores cap downwards in 40 ml. distilled water in a crystallising dish for twenty four hours was also sterilised by filtration. Aliquots of 1 ml of a washed suspension of V. malthousei spores were mixed with equal volumes of the extracts, after which was added 1 ml. water or mushroom exudate as detailed in Table 26. After incubation for fifteen hours at 24 C on glass slides, the percentage germination of spores was determined, at least two hundred spores being counted in the germination assay. The experiment was repeated twice except that part of the extracts of non-sterilised casing were heated at 100 C for thirty minutes. The results for all three experiments are listed in Table 26.

The germination rate of V. malthousei conidia in extracts of

Table 26 (part 1) Germination of *V. malthousei* conidia in soil extracts in three experiments

Treatments: Extracts from:-	Addition	No. spores germinated	No. spores ungerminated	Percentage and mean % germination	
Non sterile soil	Water	38	138	21.6	
"	"	99	221	30.9	19.5
"	"	14	223	5.9	
"	Mushroom exudate	122	32	79.2	
"	" "	195	58	77.1	81.8
"	" "	219	27	89.0	
" (heated)	Water	145	17	95.0	a.95.0
" "	"	84	136	38.2	b.38.2
" "	"	-	-	- *	
Non sterile peat	Water	100	79	55.8	
"	"	93	143	39.4	45.7
"	"	100	138	42.0	
"	Mushroom exudate	124	43	74.3	
"	" "	156	60	72.2	72.2
"	" "	152	65	70.0	
" (heated)	Water	139	23	85.8	a.85.8
" "	"	94	161	36.9	b.39.0
" "	"	91	131	41.0	b.

a. Heated to 121 C for 60 minutes. b. Heated to 100 C for 30 minutes.
* Discarded due to dehydration.

Table 26 (part 2) Germination of *V. malthousei* conidia in soil extracts in three experiments

Treatments		No. spores	No. spores	Percentage and mean%	
Extracts from :-	Addition	germinated	ungerminated	germination	
Sterile soil	Water	162	32	83.5	
" "	"	113	106	51.6	67.6
" "	"	170	81	67.7	
" "	Mushroom exudate	150	29	83.8	
" "	"	197	30	86.8	86.6
" "	"	281	26	89.2	
Sterile peat	Water	141	18	88.7	
" "	"	105	111	48.6	74.6
" "	"	184	29	86.4	
" "	Mushroom exudate	100	19	84.0	
" "	"	174	31	84.9	88.3
" "	"	198	8	96.1	

unsterilised soil was considerably increased by mushroom sporophore exudate supporting earlier suggestions of release of metabolites by mushroom hyphae in casing (see Tables10,11 and 12). The results from peat are similar but less marked. Extracts of non-sterile soil and peat heated to 100 C for thirty minutes support less germination than extracts heated to 121 C for sixty minutes or extracts of sterilised soil, suggesting that severe heat treatment is required to break down fungistatic compounds or to release sufficient nutrients to support spore germination.

The variable results obtained with water extracts of soil support the conclusion by Lockwood (1964) that this method is inherently unsuitable for examining soil fungistasis. Although Jackson (1958) and Dobbs, Henson and Bywater (1960) considered that the agar disc method of detecting soil fungistasis was less sensitive than glass slide and 'cellophane' techniques, Lockwood and Lingappa (1963) concluded that the methods were comparable. Since the agar block technique is the most reproducible one in practice, it was applied to V. malthousei spores.

c. The use of agar blocks to study spore germination in casing materials.

Two discs 7.5 x 1.5 mm. were cut from petri-dishes containing 10 ml. plain agar (1.2% Oxoid ion-agar) and placed on 2 x 2 cm. squares of filter paper on the surface of fresh soil (2 mm. sieved, 80% of maximum water-holding capacity) in crystallizing dishes. Other blocks were similarly placed on sterilised soil and on sterile

and non-sterile peat casing materials. Two further blocks were placed on a sterile glass slide on the surface of soil or peat in each dish. After four hours 0.03 ml. of a suspension of V. malthousei conidia was added to each block and the percentage spore germination was determined after twenty four hours incubation at 24 C. The results (Table 27) show that the germination rate is considerably reduced on blocks in contact with non-sterile

Table 27 Germination of V. malthousei conidia on blocks incubated on soil and peat.

Treatment of blocks	No. spores germinated	No. spores ungerminated	Percentage germination
Non-sterile soil	77	129	37.4
Non-sterile peat	109	161	40.4
Sterilised soil	147	61	70.7
Sterilised peat	162	83	66.1

peat and soil. The absence of volatile inhibitors was indicated by high rates of germination of spores incubated on blocks on glass slides in the crystallising dishes. Further work showed that blocks placed on soil for four to twenty four hours and then removed for germination assay on glass slides produced similar results (Table 28, Fig. 9). All further work with agar blocks on soil was carried out after twenty four hours exposure to soil

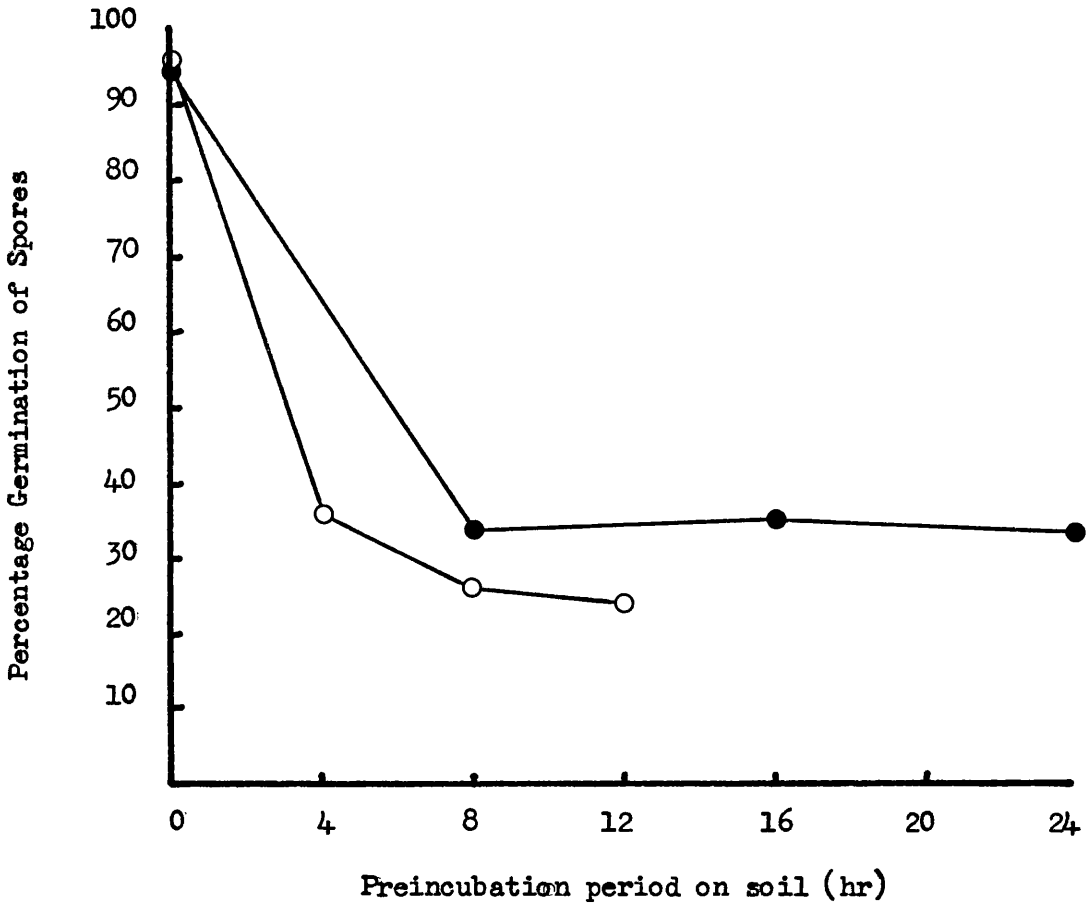


Fig.9 Germination of V. malthousei on agar blocks preincubated on soil for different periods.

Table 28 Germination of conidia of V. malthousei on plain agar blocks preincubated on soil for various time intervals.

Incubation on soil (hours)	No. spores germinated	No. spores ungerminated	Percentage germination
0	193	11	94.6
8	80	157	33.8
16	81	148	35.4
24	69	139	33.2
0	195	8	96.1
4	86	152	36.1
8	58	165	26.0
12	60	191	23.9

followed by removal of blocks for germination assay unless otherwise stated.

The removal of blocks from soil before spores were added indicates that fungistasis does not depend on a continuous production of inhibitors or a continuous diffusion of nutrients into soil (Lockwood 1964) during the germination incubation period, but once the process has reached equilibrium, the effect can be assayed in the absence of soil.

d. The effect of dialysis of plain agar blocks on spore germination

If nutrients present in agar blocks on soil diffuse out, thus

depleting the agar of exogenous nutrients essential for spore germination, similar results would be expected using agar blocks freed of such nutrients by dialysis (Lockwood 1964). Distilled water was circulated for one hour, through a length of washed 'Visking' dialysis tubing, 40 x 2 cm., joined by plastic tubing to a 6 L reservoir, by a propellor-type pump having only stainless steel immersed parts. The distilled water was changed, circulation recommenced and plain agar blocks were placed on the tubing for twenty four hours in an atmosphere of high humidity to prevent drying. Two of these blocks were then each placed on a fresh cut button mushroom, filter papers soaked in 2% glucose solution and glass slides maintained in humidity chambers. After twenty four hours all blocks were placed on individual glass slides in damp chambers and a spore suspension of V. malthousei added. The results recorded in Table 29 show that the removal of diffusible nutrients by dialysis caused a great reduction in the rate of spore germination which is restored by contact with a mushroom sporophore and to a lesser degree by 2% glucose solution, providing support for Lockwood's hypothesis that fungistasis as demonstrated in agar blocks could be caused by lack of nutrients.

To determine if the rate of spore germination depended on the length of the period of dialysis, blocks were dialysed for a range of time intervals and transferred to glass slides for a spore germination assay as before. The results are shown in Table 30 and Fig. 10. Similar values were obtained using a continuous flow

Table 29. Germination of *V. malthousei* conidia on dialysed plain agar blocks.

Treatment	No. spores germinated	No. spores ungerminated	Percentage germination
Dialysed block	6	213	2.7
Dialysed block on mushroom	268	5	98.2
Dialysed block on 2% glucose	149	132	53.0
Undialysed block	192	46	80.7

Table 30 Germination of conidia of *V. malthousei* on plain agar blocks dialysed with distilled water for various time intervals.

Dialysis period (Hours)	No. spores germinated	No. spores ungerminated	Percentage germination
0	240	12	95.2
1	170	33	83.7
2	94	153	38.0
3	134	189	41.5
4	77	162	32.2
5	22	189	10.4
6	23	203	10.2
7	6	206	2.8
8	18	199	8.3
9	11	219	4.8
10	9	217	4.0

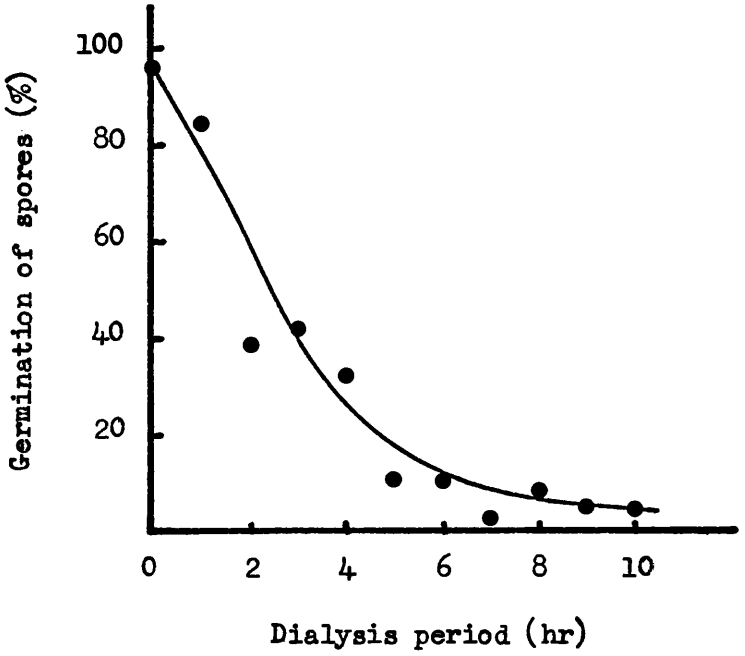


Fig.10 Germination of V. malthousei conidia on plain agar blocks dialysed with recirculated distilled water for various time intervals.

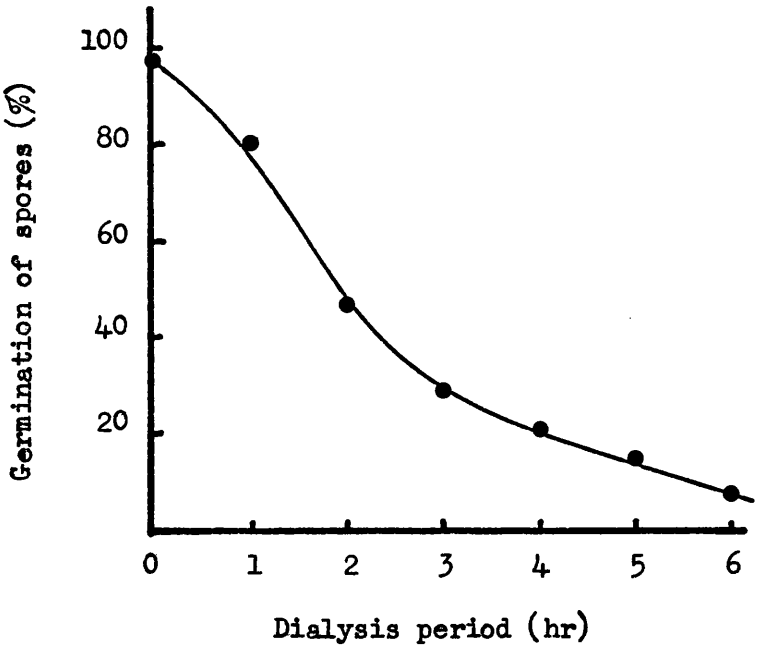


Fig.11 Germination of V. malthousei conidia on plain agar blocks dialysed with tap water for various time intervals.

of tap water in place of recirculated distilled water (Table 31, Fig.11).

Table 31 Germination of conidia of V. malthousei on plain agar blocks dialysed with tap water for various time intervals

Dialysis time (hours)	No. spores germinated	No. spores ungerminated	Percentage germination
0	203	6	97.1
1	207	52	79.9
2	93	108	46.3
3	63	158	28.5
4	46	171	21.2
5	30	179	14.4
6	20	254	7.3

The progressive reduction in the rate of spore germination with continued dialysis in similar after four hours to the values obtained for agar blocks placed on soil for various time intervals (see Table 28, Fig. 9) although further dialysis results in a greater reduction in germination.

e. The effect of nutrients on spore germination on agar blocks

To further examine dialysed agar or agar blocks exposed to soil for loss of nutrients, these blocks were placed on plain agar or dialysed plain agar discs for one hour prior to the application

of spores and allowed to remain during the incubation period. Table 32 gives the details of treatments together with the spore germination values. The experiment was repeated twice with similar results.

Table 32 Germination of *V. malthousei* conidia on plain agar blocks

No.	Treatment of plain agar blocks	No. spores germinated	No. spores ungerminated	Percentage germination
1	Dialysed block	109	99	52.4
2	Dialysed block placed on block	191	11	94.6
3	Dialysed block from soil	109	102	51.7
4	Dialysed block from soil placed on block	189	11	94.5
5	Dialysed block from soil placed on dialysed block	80	123	39.4
6	Block from soil	113	116	49.3
7	Block from soil placed on block	203	13	94.0
8	Block from soil placed on dialysed block	98	104	48.5

Clearly materials diffusing from plain agar can overcome soil fungistasis in blocks exposed to soil (treatments 4 and 7). If inhibitory materials diffusing from soil into blocks are the only factors involved in fungistasis then treatment 3 should show reduced germination compared with controls, while the redistribution of such materials might be expected to influence the result in treatment 5 which showed the lowest percentage germination. The absence of such effects in conjunction with other results obtained underline the relationship between germination and availability of nutrients in soil fungistasis.

To obtain an indication of the quantity of nutrients required to support spore germination, an assay was carried out to determine the range of glucose concentrations required to promote germination of washed conidia of V. malthousei. These were suspended in distilled water containing 0.01 - 1000 ppm 'Analar' dextrose on glass slides at 24 C for fifteen hours (Table 33, Fig. 12).

Table 33 Germination of conidia of V. malthousei in glucose solutions

Glucose concentration (ppm)	No. spores germinated	No. spores ungerminated	Percentage germination
0	36	121	22.9
0.01	46	135	25.4
0.1	52	105	33.1
1.0	-	-	- *
10.0	85	108	44.0
100.0	78	61	56.1
1000.0	110	98	52.9
Malt agar	245	13	95.0

* Discarded due to dehydration

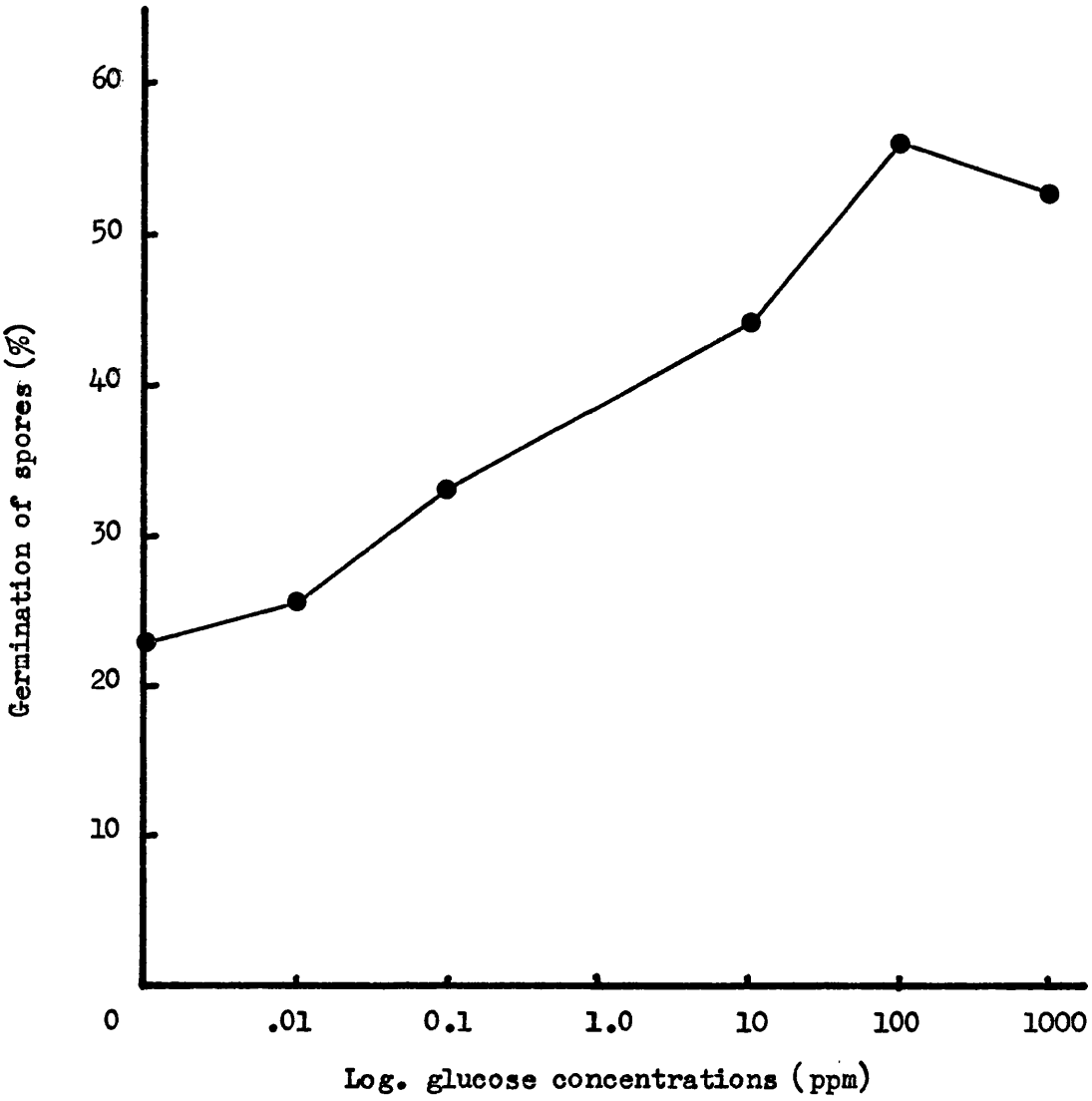


Fig.12 The effect of glucose on the germination of V. malthousei conidia.

Similar results were obtained in repeated experiments when dialysed plain agar blocks, after exposure to soil, for 0 - 24 hours, were floated in 0.1 ml. glucose solutions on glass slides (Table 34, Fig. 13). When these combined results were subjected to an analysis of variance, the differences between the four treatments were found not significant at the 5% level of probability using the variance-ratio test. Glucose concentrations of 10 ppm. and above consistently increased the rates of germination irrespective of the duration of exposure to soil, which is difficult to explain if fungistatic factors are present in the agar, since glucose concentrations required to annul fungistasis in dialysed blocks exposed to soil should be higher than those required by dialysed agar.

f. Nutrient content of extracts of agar blocks and soil.

While dialysis experiments provide supporting evidence for the interpretation of soil fungistasis in terms of deficiency of nutritional factors necessary for spore germination, further pre-requisites for the acceptance of this concept are proof of removal of such materials from plain agar when exposed to soil and the enrichment of the nutritional status of soil during sterilisation. Evidence along these lines was sought by determination of the hexose content of water extracts of plain agar and soil, as an index of nutrient status, using the phenol-sulphuric acid colourimetric method (Whistler and Wolfrom 1962).

Analytical grade reagents and 'Decon' washed, dust-protected

Table 34*

Germination of V. malthousei conidia on dialysed plain agar blocks treated with glucose solutions after incubation on soil

Glucose conc. (ppm)	Preincubation period on soil (hr) 0	8	16	24
0	22.0	21.8	18.4	19.9
0.01	31.1	17.0	34.4	31.7
0.1	19.5	34.8	22.2	32.0
1.0	24.7	35.5	43.3	35.4
10	54.1	56.3	71.2	53.4
100	60.3	74.5	72.0	83.9
1000	61.7	76.0	75.2	84.7

* For details see Appendix 2, Table 46.

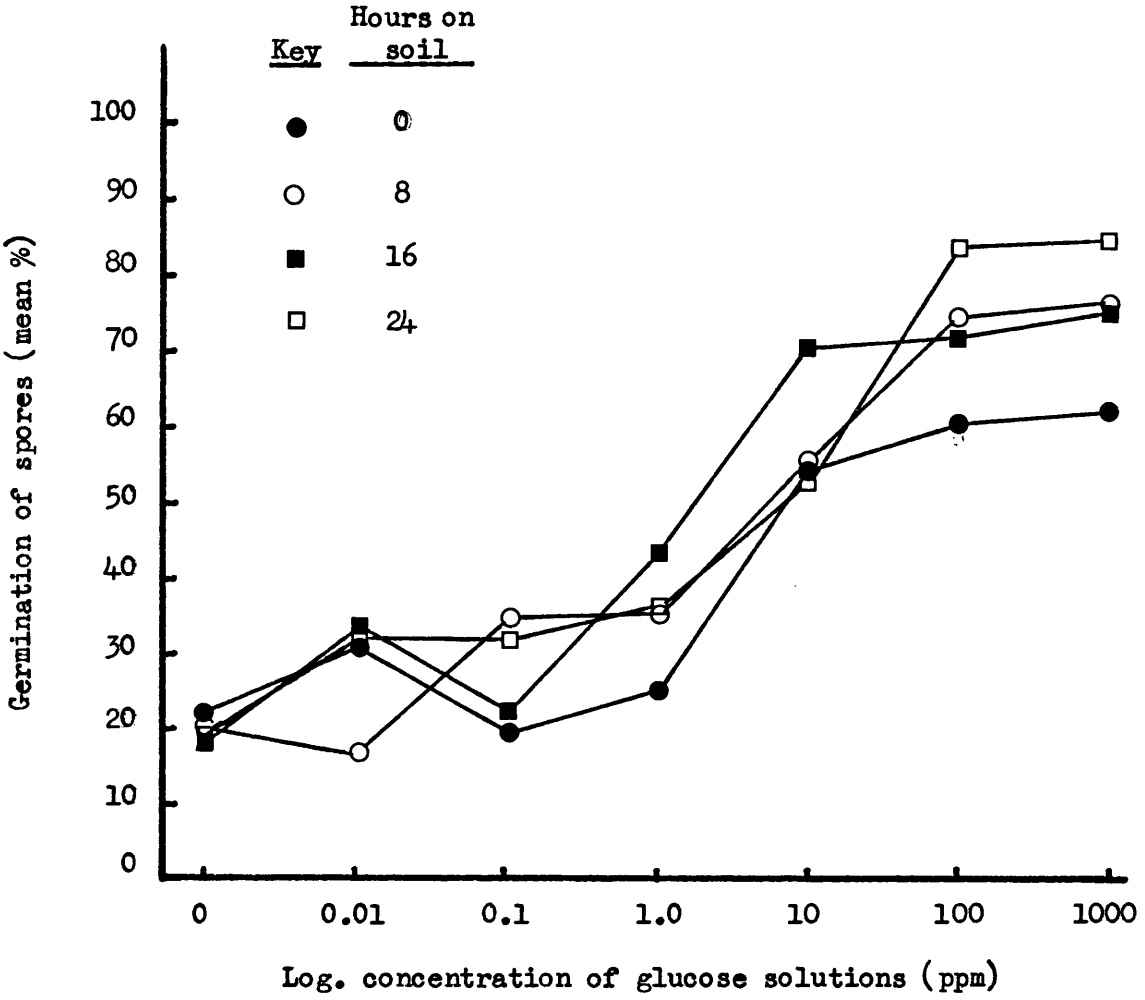


Fig.13 Germination of V. malthousei conidia on dialysed plain agar blocks treated with glucose solutions after incubation on soil for different periods.

glassware were used throughout. Hexose concentrations were measured against glucose standards and were expressed as equivalent to glucose concentrations. The method measured over the range 10 - 70 µg glucose/ml with an accuracy of ± 2% (Whistler and Wolfrom 1962). To measure concentrations less than 10 µg/ml., each aliquot of extract was augmented with 20 µg glucose/ml (e.g. Fig. 14). The test solutions were suitably diluted to contain 0 - 50 µg/ml. Aliquots of 1 ml. were pipetted into boiling tubes and mixed with 1 ml of a solution containing 5% phenol and 20 µg/ml glucose. Each tube was agitated in a standard manner after 5 ml 96% sulphuric acid was added. The tubes were allowed to cool to room temperature for sixty minutes and the absorbances were read at 490 µ on a Unicam SP600 spectrophotometer. All determinations were carried out in triplicate to allow exclusion of anomalies due to accidental contamination (Oades 1967) and the results averaged. The concentration of hexoses in the test solution was obtained as the glucose equivalent by reference to the standard curve (e.g. Fig.14).

Air-dried soil (10g.) of particle size up to 2 mm, which had been stored for several months, was placed in each of two screw-cap bottles and heated to 121 C for thirty minutes in an autoclave, after which 2 ml distilled water was added. A similar volume of soil kept moist for four weeks previously was placed in each of two more bottles and 10 ml distilled water added. All soils were shaken vigorously for thirty minutes on a mechanical wrist-action shaker, centrifuged twice and 5 ml of the supernatant

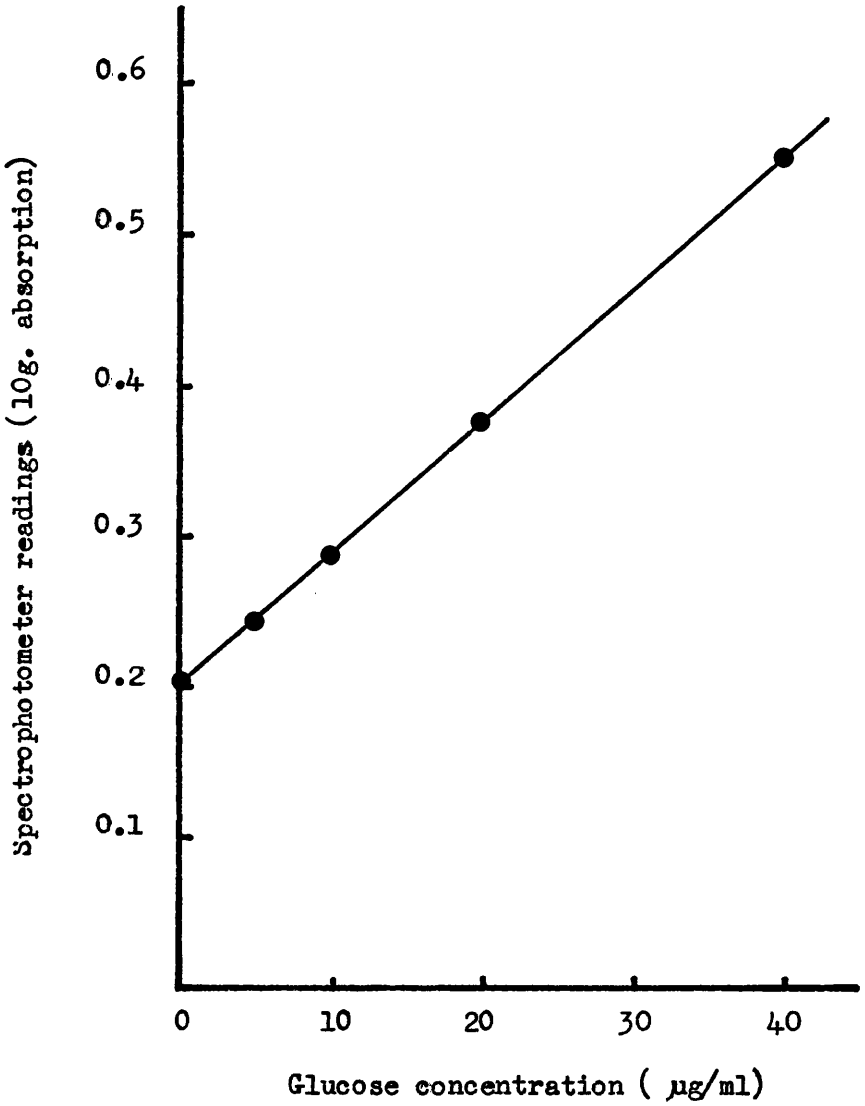


Fig.14 Specimen standard curve from analysis of standard solutions of glucose, using phenol-sulphuric acid method with phenol solution augmented with 20 µg/ml glucose.

sterilised by membrane filtration. Four tenfold dilutions of the extracts were made and the concentrations of soluble hexoses were determined. The experiment was repeated twice, heating dried soil for fifteen minutes only and using soil moistened for five and six weeks respectively. In addition dry stored soil was extracted without heat treatment and soils moistened prior to the experiment were extracted after heat treatment. The results in Table 35 show that sterilisation of dry or moist soil increases the content of soluble hexoses measured as glucose, confirming the observations of Ko and Lockwood (1967). Extracts of dry stored soil also had a greatly increased nutrient content compared with moist soil.

Extracts of agar blocks used in the detection of soil fungistasis were next examined for soluble hexose content. Plain 'ion-agar' discs were dialysed over tap water and placed in groups of six in crystallizing dishes on washed dialysis membranes covering sterilised or untreated moist soil. Care was taken to ensure that no bubbles were trapped between the agar and membrane and intimate contact between membrane and soil was achieved by covering the blocks with a small inverted petri-dish, pressing the membrane into the soil (Fig.15). Undialysed agar discs were also placed over sterilised and natural soil and all treatments were incubated for twelve hours at 24 C. Four discs from each treatment together with untreated dialysed and undialysed blocks were carefully transferred to boiling tubes containing 4 ml distilled water and gently

Table 35

Soluble hexose content of soil extracts

Soil	Heat treatment		Hexose content of extract ≡ µg/ml glucose
	Temperature (C)	Time (min)	
Moist for 4 weeks	-	-	21
" " 5 "	-	-	18
" " 6 "	-	-	24
Dry stored	-	-	320
Dry stored	121	15	610
" "	121	15	640
" "	121	30	950
Moistened prior	100	30	1300
to heating	121	30	1700

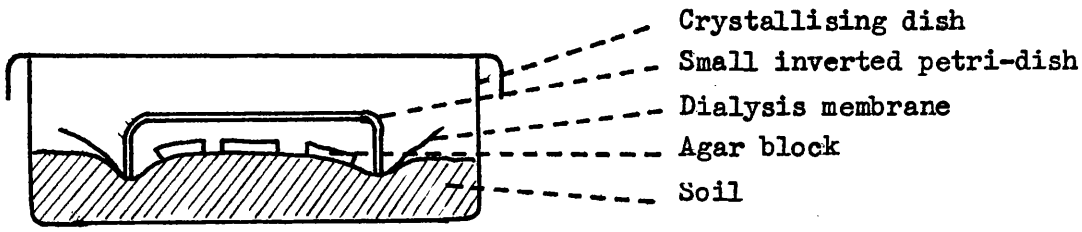


Fig.15 Diagram of agar blocks on a dialysis membrane over moist soil.

agitated for thirty minutes. The water extracts were decanted, sterilised by membrane filtration and soluble hexoses determined. The remaining two blocks from each treatment were placed on glass slides for a spore germination assay. The experiment was repeated and the results are shown in Tables 36 and 37 and Fig. 16. A second repeat was carried out with the blocks incubated on soil for twenty four hours, and the extracts determined without membrane filtration (Table 38, Fig. 17). The coefficients of correlation between spore germination and the hexose content of the extracts listed in Tables 36, 37 and 38 were 0.79, 0.91 and 0.89 at a significance level of 90%, 98% and 98% respectively (four

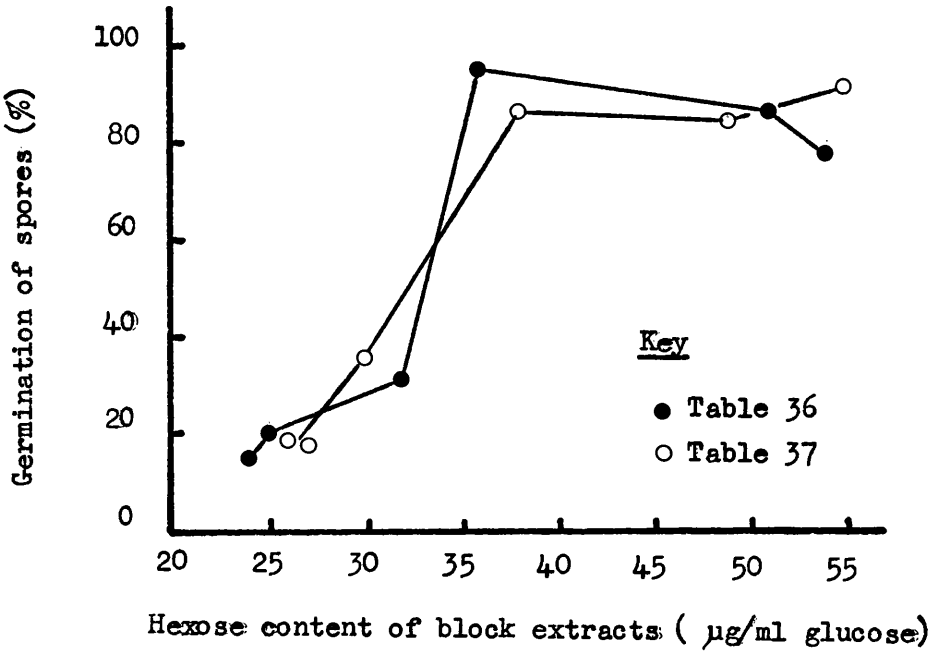


Fig.16 Germination of V. malthousei conidia on agar blocks and hexose content (equivalent to glucose) of sterile-filtered water extracts of the blocks.

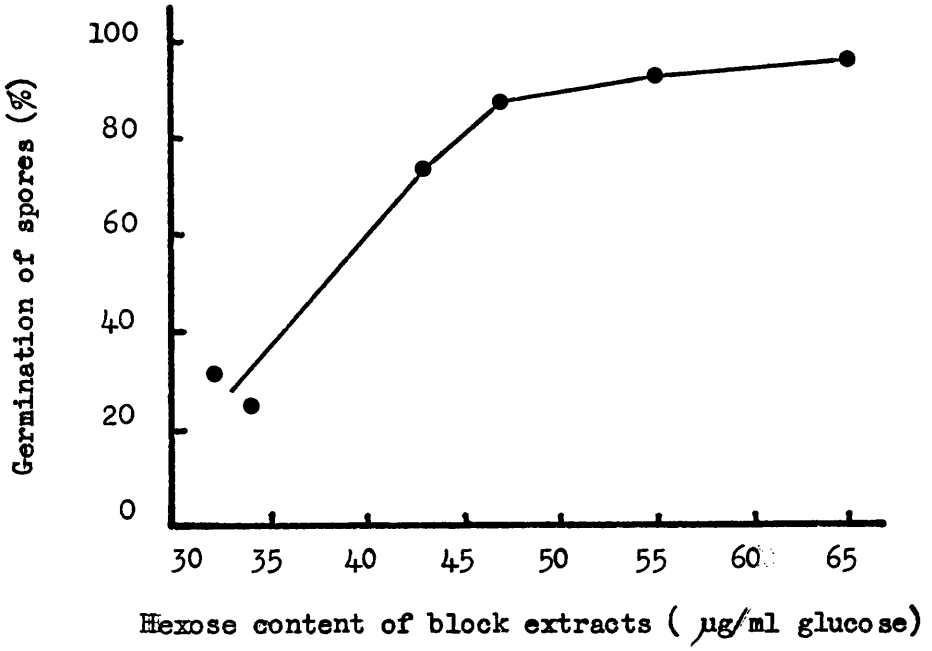


Fig.17 Germination of V. malthousei conidia on agar blocks and hexose content (equivalent to glucose) of unsterilised water extracts of the blocks

Table 36

Germination ov *V. malthousei* conidia on agar blocks and the hexose content of sterilised block extracts

Treatment	No. spores germinated	No. spores ungerminated	Percentage germination	Hexose content of extract µg/ml
Dialysed agar	37	216	14.6	24
Undialysed agar	196	10	95.1	36
Dialysed agar on sterile soil	263	81	76.5	54
Undialysed agar on sterile soil	179	28	86.4	51
Dialysed agar on natural soil	48	188	20.3	25
Undialysed agar on natural soil	106	233	31.3	32

Table 37 Germination of *V. malthousei* conidia on agar blocks and the hexose content of sterilised block extracts

Treatment	No. spores germinated	No. spores ungerminated	Percentage germination	Hexoses in extracts µg/ml
Dialysed agar	51	229	18.2	26
undialysed agar	231	37	86.1	38
Dialysed agar on sterile soil	251	47	84.3	49
Undialysed agar on sterile soil	222	23	90.8	55
Dialysed agar on natural soil	44	211	17.7	27
Undialysed agar on natural soil	126	238	35.3	30

Table 38 Germination of *V. malthousei* conidia on agar blocks and the hexose content of unsterilised block extracts

Treatment	No. spores germinated	No. spores ungerminated	Percentage germination	Hexoses in extracts µg/ml
Dialysed agar	109	329	24.4	34
Undialysed agar	189	29	86.7	47
Dialysed agar on sterile soil	212	19	91.8	55
Undialysed agar on sterile soil	198	10	95.2	65
Dialysed agar on natural soil	108	193	35.9	32
Undialysed agar on natural soil	415	146	73.4	43

degrees of freedom).

Several points emerge from these experiments. In each treatment low germination rates were paralleled by low values for soluble hexoses extracted. The concentration of these materials appears to be critical over the range 32 - 36 µg/ml. for Tables 36 and 37 and 34 - 43 µg/ml. for Table 38 using a different method. While dialysed and undialysed agar took up soluble hexoses from sterilised soil, confirming the results obtained for soil extracts (see Table 35), dialysed agar was not enriched sufficiently to support spore germination when placed on natural soil. Undialysed agar was depleted of soluble hexoses when placed on soil, sufficiently in Table 36 and 37 to markedly reduce the rate of spore germination. In each treatment, spore germination values are reflected in the hexose concentration of the extracts. The results of this work clearly show that spore germination in soil and on agar blocks used for the detection of fungistasis, is controlled by the level of exogenous nutrients. While the presence of a diffusible microbially-produced inhibitor has not been refuted, no evidence for its' existance has been produced in any experiment. Thus the behaviour of V. malthousei in casing materials can be explained by the majority of conidia requiring exogenous nutrients for germination which are not supplied by soil or peat.

7. CHEMICAL CONTROL OF V. MALTHOUSEI

Following the recommendations of Sinden and Yoder (1949), zineb and latterly other dithiocarbamate fungicides, have become established in the mushroom industry for the control of V. malthousei and M. perniciosa. However, a survey (Gandy 1957) showed that although well over half the growers whose farms are infected with these pathogens use dithiocarbamates, only a quarter of them achieve adequate control of disease. Kneebone and Merek (1961) recommended an increased frequency of fungicide application although Yoder et al (1950) and Philipp (1963) had recorded yield reductions due to fungicide. From the results of a questionnaire Last and Gandy (1965) concluded that the control of M. perniciosa was associated with good hygienic practice, the dithiocarbamate fungicides being ineffective when used prophylactically, but may be of some value in the treatment of diseased mushrooms before their removal from the beds.

Possible reasons for this apparent lack of effectiveness may be inactivation of the fungicide in casing, an insufficient concentration of fungicide being used or the aquisition of resistance by the pathogens. No published toxicity data for zineb against the mushroom and pathogens exist. Comparative laboratory data is also lacking on the effects of zineb and the newer dithiocarbamate compounds coming into use. The possibility of aquired resistance to zineb by the mushroom pathogens has not been investigated.

In the present study, an examination was made with V. malthousei

of the effectiveness of zineb in preventing infection of mushrooms in laboratory *in vivo* tests, the possibility of aquired resistance, the toxicity of zineb to spores in soil and to determine whether zineb exerts a sporicidal or sporostatic action. In addition the stability of zineb was examined, in particular for possible inactivation in soil. The effect of zineb and other selected dithiocarbamate fungicides on mycelial growth of *V. malthousei* and *A. bisporus* was also determined. In view of the work of Wuest and Cole (1970) benomyl (methyl 1 - (butylcarbamoyl) - 2 - benzimidazolecarbamate) ~~are~~ is included in this work.

a. *The Effectiveness of Zineb*

The value of zineb in preventing infection of mushrooms by *V. malthousei* was examined in laboratory *in-vivo* experiments. Spawn-run compost placed in five inch diameter plastic flower pots was cased with a 2 cm. layer of moist peat with limestone chippings and each of ten pots sprayed with 16.6 ml. of a 0.1% suspension of fresh Vitax 65% zineb wettable powder, which is in accordance with a commercial recommended rate (Sinden and Yoder 1949). Further groups of pots were sprayed with a suspension containing 10 and 100 times this concentration, a control treatment being sprayed with water. Each pot was then inoculated with 4 ml. of a suspension of spores of *V. malthousei* containing 2×10^6 spores/ml and incubated in the laboratory in a ventilated polythene chamber at 19 - 25 C and 90 - 100% relative humidity. A further series of

pots was inoculated with spores three days after being treated with fungicide. Watering was carried out by sprinkling, precautions being taken to prevent cross-infection, the numbers of healthy and diseased mushrooms being recorded from each pot for 2 - 3 flushes. The experiment was repeated and the combined results shown in Table 39 indicate that satisfactory disease control is not achieved at the commercially recommended rate used.

Table 39

Numbers of healthy and diseased mushrooms in pot culture with zineb fungicide treatments.

Zineb concentration*	Spores and Fungicide added together		Spores added 3 days after fungicide	
	Healthy	Diseased	Healthy	Diseased
0	37	22	38	61
1	44	25	24	59
10	65	12	38	59
100	48	7	37	19

*Key 1 = Rate recommended by Sinden and Yoder (1949)
10 = 10 x " " " " " "
100 = 100 x " " " " " "

At 10 times and 100 times such rates of application, disease levels are reduced, particularly when spores are added immediately after the fungicide.

b. Resistance to zineb in V. malthousei

To examine the possibility of aquired resistance to zineb, isolates of V. malthousei were taken from diseased mushrooms from farms with differing fungicide application programmes. On all the farms, disease caused by V. malthousei had been observed over many years and it is reasonable to assume that resistance will be more likely to develop in pathogens isolated from farms where zineb has been used regularly over a long period of time. The isolates employed were as follows :-

Isolate (W) was obtained from Wrington Vale Nurseries, Ltd., Congresbury, Somerset. Zineb was used on this farm until 1967 when this isolate was obtained.

Isolate (X) was obtained from Sampson Mushrooms Ltd., Oving, Chichester, Sussex where zineb was used for many years up to the time of isolation.

Isolate (Y) was obtained from Wrington Vale Nurseries Ltd., Buxton Derbyshire where no dithiocarbamates were used over a three year period prior to isolation.

Isolate (Z) was obtained from Agarie Ltd., Bradford-on-Avon, Wiltshire where no dithiocarbamate fungicides were used for many years up to the time of isolation.

The effect of zineb on the germination of spores of these isolates was taken as the basis for determining resistance. The slide germination technique was used (Anon 1947). All glassware was washed, soaked in 'Decon', throughly rinsed in tap then

distilled water and oven-dried prior to use except pipettes which were cleaned in chromic acid followed by continuous rinsing in tap water for at least twelve hours. Serial dilutions of zineb were made in distilled water, the fungicides being maintained in suspension during dilution by appropriate agitation. All concentrations were based on the percentage active ingredient of the fungicide in the commercial products used. For each organism and each concentration, 2 ml. diluted fungicide suspension were placed in a test tube. To ensure maximal germination of spores an orange juice stimulant was prepared by filtering the juice of good quality oranges through cheesecloth, filter paper and cellulose acetate membrane filters (Oxoid). Following tenfold dilution with distilled water, the resulting clear filtrate was distributed in 10 ml. aliquots into bijou bottles and stores in a deep freeze cabinet at -20 C. When required, the contents of one bijou bottle were diluted to 100 ml. with distilled water, the concentration of orange juice in the final spore suspension being 0.1%.

The fungi to be tested were isolated and maintained on malt extract agar (Oxoid). Washed spore suspensions prepared as described previously were mixed with an equal volume of germination stimulant and 0.5 ml. aliquots were pipetted into test tubes containing 2 ml. diluted fungicide and two separate droplets were placed on slides which were incubated in individual damp chambers at 24 C for twenty four hours. After incubation the percentage germination for at least two hundred spores at each concentration was determined

and the percentage germination inhibition for each treatment was calculated, based on controls corrected to 100% germination.

Spore germination inhibition values for the four isolates in response to zineb concentrations 0 - 100 ppm are listed in Table 40 and Fig.18, a further experiment indicating that germination was completely inhibited at 1000 ppm. When the data from this former work were subjected to an analysis of variance, differences between the isolates were not significant at the 1% level of probability, using the variance-ratio test. These results show the similarity in toxicity of zineb to the four isolates (LD_{50} 35.9, 41.7, 44.7, 45.7 ppm for isolates W, X, Y and Z respectively, mean 42.0 ppm) and suggest there is no evidence of aquired resistance to zineb in the strains tested, these observations supporting the views of Horsfall and Lukens (1966) that dithiocarbamates do not induce resistance in fungi.

c. Toxicity of zineb to V. malthousei in soil

To determine whether the ineffectiveness of zineb at controlling disease at standard application rates was due to lack of toxicity of the fungicide in the casing, an assay was carried out with V. malthousei conidia supported on glass slides in soil. Zineb was thoroughly mixed with 100 g. quantities of dry soil at 0, 45, 75, 450 and 750 ppm active ingredient (w/w, zineb/soil) and the soils made up to 50% of maximum water-holding capacity. The soil was pressed down on glass slides on which had been spread

Table 40

The effect of zineb suspensions on spore germination of isolates of V. malthousei

Isolate	Zineb conc. (ppm)	No. spores germinated	No. spores ungerminated	Corrected % spore germination inhibition
(W)	0	228	7	0
	20	226	25	7.2
	40	93	114	55.8
	60	81	145	63.1
	80	63	146	69.0
	100	44	180	79.8
(X)	0	205	4	0
	20	212	13	4.0
	40	106	104	48.5
	60	84	134	60.8
	80	72	136	64.7
	100	23	193	89.2
(Y)	0	211	7	0
	20	186	32	11.9
	40	113	103	46.0
	60	88	131	58.5
	80	69	146	66.8
	100	34	177	83.4
(Z)	0	198	9	0
	20	177	31	11.1
	40	106	102	46.7
	60	104	149	56.7
	80	75	135	62.7
	100	44	177	79.2

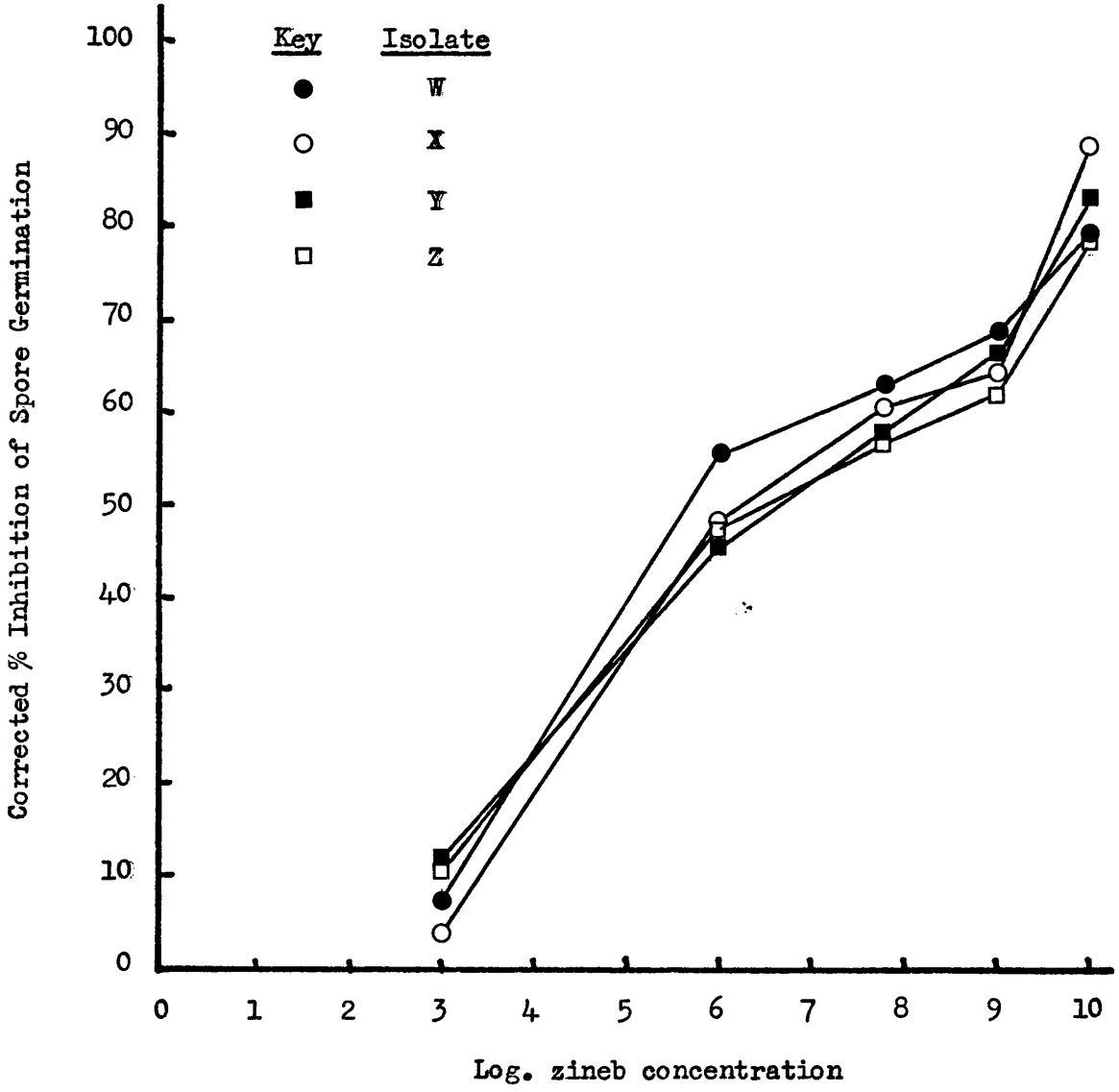


Fig. 18 Corrected spore germination inhibition (%) of V. malthousei in the presence of zineb

a spore suspension of V. malthousei augmented with sufficient nutrients to ensure adequate germination in soil. After twenty four hours incubation in petri-dishes in a damp chamber, the soil was tipped off, the slides dried over a lamp and further excess soil particles removed. After staining with acid fuschin in lactophenol, the slides were examined using normal and phase contrast microscopy. The results of two experiments combined in Table 41 indicate that the fungicide is effective at completely inhibiting spore germination at 750 ppm in soil. In the infection

Table 41 Germination of V. malthousei conidia in soil treated with zineb

Zineb conc. (ppm soil)	No. spores germinated	No. spores ungerminated	% spore germination	Mean corrected % germination inhibition
0	232	29	88.9	0
	249	22	91.8	
45	229	18	84.5	0.9
	227	13	94.6	
75	218	7	96.8	0
	242	26	90.3	
450	14	213	6.2	95.3
	5	203	2.4	
750	0	200	0	100.0
	0	200	0	

experiment described earlier, the amount of fungicide applied at the standard rate was 0.082 mg/cm^2 which is equivalent to a soil concentration of 750 ppm if evenly distributed through only approximately 1.3 mm. soil (bulk density of soil 1.17). If this is comparable with peat and limestone casing materials, effective disease control should be achieved if zineb were confined to the surface or distributed within the upper 1.3 mm. of casing.

d. The mode of action of zineb

From the data already presented it is not possible to determine whether the effect of zineb on conidia of V. malthousei is sporostatic or sporicidal. Thus an experiment was performed using zineb in malt agar to examine this effect. V. malthousei spore suspensions were filtered through Oxoid membrane filters which were in turn placed on malt agar plates containing 0 - 10,000 ppm zineb, mixed with cooled agar prior to pouring. After twenty four hours the filters were cut in half and one half placed on fresh malt agar plates containing no fungicide for a further twenty four hours incubation. Spore germination values for the critical concentrations only for this and a repeat experiment shown in Table 42, indicate that approximately 30% of spores inhibited at the 10,000 ppm level, subsequently germinated on removal from the fungicide. A further group of spores on membranes, incubated in contact with fungicide for forty eight hours showed that those on the 10,000 ppm remained largely ungerminated as in Table 42. Thus is is concluded

that at this concentration and for this exposure period, zineb exerts a sporostatic effect on about 30% of spores, the remainder being presumed killed.

Table 42 Germination of V. malthousei conidia on membrane filters in contact with zineb in agar, and after removal from fungicide.

Zineb conc. in agar (ppm)	No. spores germinated	No. spores ungerminated	% germination
	Incubation in contact with fungicide		
0	213	3	98.6
	267	5	98.2
1000	275	19	93.5
	228	16	93.4
10000	2	298	1.5
	3	242	1.2
	Incubation after removal from fungicide		
10000	50	153	24.6
	123	227	35.1

e. Stability of Zineb

The inhibitory effect of zineb over several days was assayed using spore germination inhibition in V. malthousei as the criterion

of toxicity. Fungicide stability in water was examined by allowing zineb suspensions 0 - 10,000 ppm to stand for 0, 3 and 6 days at 20 - 24 C followed by a spore germination assay. The experiment was repeated and the results indicate that little loss in activity occurs during this time, the germination rate at the 100 ppm level being reduced less than 10% maximum (7% mean) over six days. Rather more loss in activity occurred when a similar experiment in duplicate and repeated was carried out with fungicide suspended in malt agar (Table 43). This result is particularly relevant to the method recorded below of determination of fungicide toxicity to mycelial growth on agar plates which extends over about fourteen days. When similar studies on fungicide stability were carried out in soil with zineb at 0, 45, 75, 450 and 750 ppm soil made up to 50% of maximum water-holding capacity and stored in darkness at 20 - 24 C and 100% R.H. for up to fourteen days, the increase in germination rate was less than 10% at 450 ppm (see Table 41) indicating that the inhibitory effects of zineb on spore germination are relatively stable under the conditions described.

f. Selectivity of Fungicides

The selective effects of fungicides for V. malthousei (isolate W) compared with A. bisporus (Darlingtons) were examined by determining the rate of radial growth of colonies on malt agar containing fungicide (Horsfall 1956). Two other dithiocarbamates which have recently become available were compared with zineb -

Table 43 Germination of *V. malthousei* conidia on zineb/malt agar plates 0 - 6 days old

Fungicide age (days)	Zineb conc. (ppm)	No. spores germinated	No. spores ungerminated	Germination %	Mean %
0	100	7	226	3.0	3.1
		9	223	3.9	
		4	206	1.9	
		8	206	3.7	
	1000	0	200	0	0
		0	200	0	
		0	200	0	
		0	200	0	
3	100	31	194	13.8	14.3
		31	175	15.0	
		47	245	16.1	
		29	210	12.1	
	1000	3	214	1.4	1.0
		1	273	0.4	
		2	231	0.9	
		4	285	1.4	
6	100	40	182	18.0	22.5
		48	161	23.0	
		55	178	23.6	
		75	218	25.6	
	1000	6	242	2.4	2.3
		2	271	0.7	
		10	312	3.1	
		8	262	3.0	

namely 'Vertomyc' (manganese zinc dithiocarbamate formulated as a 15% dust, supplied by Shirley Organics Limited, Maple Cross, Rickmansworth, Herts.) and 'Cufram Z' (manganese zinc copper iron dithiocarbamate formulated as an 80% wettable power manufactured by Universal Crop Protection Limited, Maidenhead, Berks.) In addition Benlate (benomyl formulated as a 50% wettable powder manufactured by Du Pont) was included in the experiment.

In each case, the fungicide powder which was found to be practically sterile, was diluted in sterile water and 1 ml incorporated in 9 ml. cooled malt extract agar in petri-dishes. Five replicate plates for each concentration were prepared with appropriate controls. Discs of culture were inoculated into the centre of each plate mycelium uppermost and incubated at 24 C in the dark at 100% relative humidity to prevent drying. The maximum and minimum diameters of each colony were measured over a period of nine days. Mean diameter measurements for each concentration were calculated (Appendix 3, Table 47) and plotted graphically against time (Appendix 3, Figs. 23 - 30). The mean radial growth rates of the colonies in mm/day were determined and expressed as a percentage of controls (Table 44, Figs. 19 - 22). Toxicity of these fungicides to V. malthousei and A. bisporus is

Table 44

Radial growth rates of colonies of V. malthousei and A. bisporus growing on agar containing fungicide

Fungicide conc. (ppm)	V. malthousei Radial growth (mm/day)	V. malthousei % of control	A. bisporus Radial growth (mm/day)	A. bisporus % of control
Zineb				
0	0.82	100.0	2.08	100.0
10	0.89	108.5	2.08	100.0
100	1.16	141.5	2.00	96.2
1000	0.92	112.2	0.25	12.0
10000	0.85	103.5	0	0
Cufram Z				
0	0.81	100.0	2.03	100.0
10	0.89	109.8	1.83	90.1
100	1.01	124.8	1.00	49.3
1000	0.84	103.6	0	0
10000	0.14	17.3	0	0
Vertomyc				
0	0.84	100.0	2.00	100.0
10	0.84	100.0	2.00	100.0
100	0.82	97.7	2.00	100.0
1000	0.83	98.8	0.08	4.0
10000	0.33	39.3	0	0
Benlate				
0	0.80	100.0	2.06	100.0
0.1	0.83	103.7		
1.0	0.79	98.7	2.00	97.1
10	0.20	25.0	0.47	22.8
50			0.01	0.5
100	0.17	21.2	0	0

expressed in ED_{50} values (50% inhibition of mycelial growth as determined by the extension of fungal colonies) which were obtained from Figs. 19 - 22 and listed in Table 45. These results show that mushroom mycelium is more sensitive to zineb and the newer dithiocarbamates than is V. malthousei, benlate having a similar effect on both organisms. This may account in part for the relative innfectiveness of zineb in the control of V. malthousei on mushroom beds.

Table 45.

ED_{50}* values of fungicide concentration (ppm) for V. malthousei and A. bisporus.

Fungicide	V. malthousei	A. bisporus
Zineb	-	363
Cufram Z	4170	100
Vertomyc	6170	316
Benlate	4.7	4.2

* ED_{50} - Fungicide concentration effective at reducing colony growth by 50%.

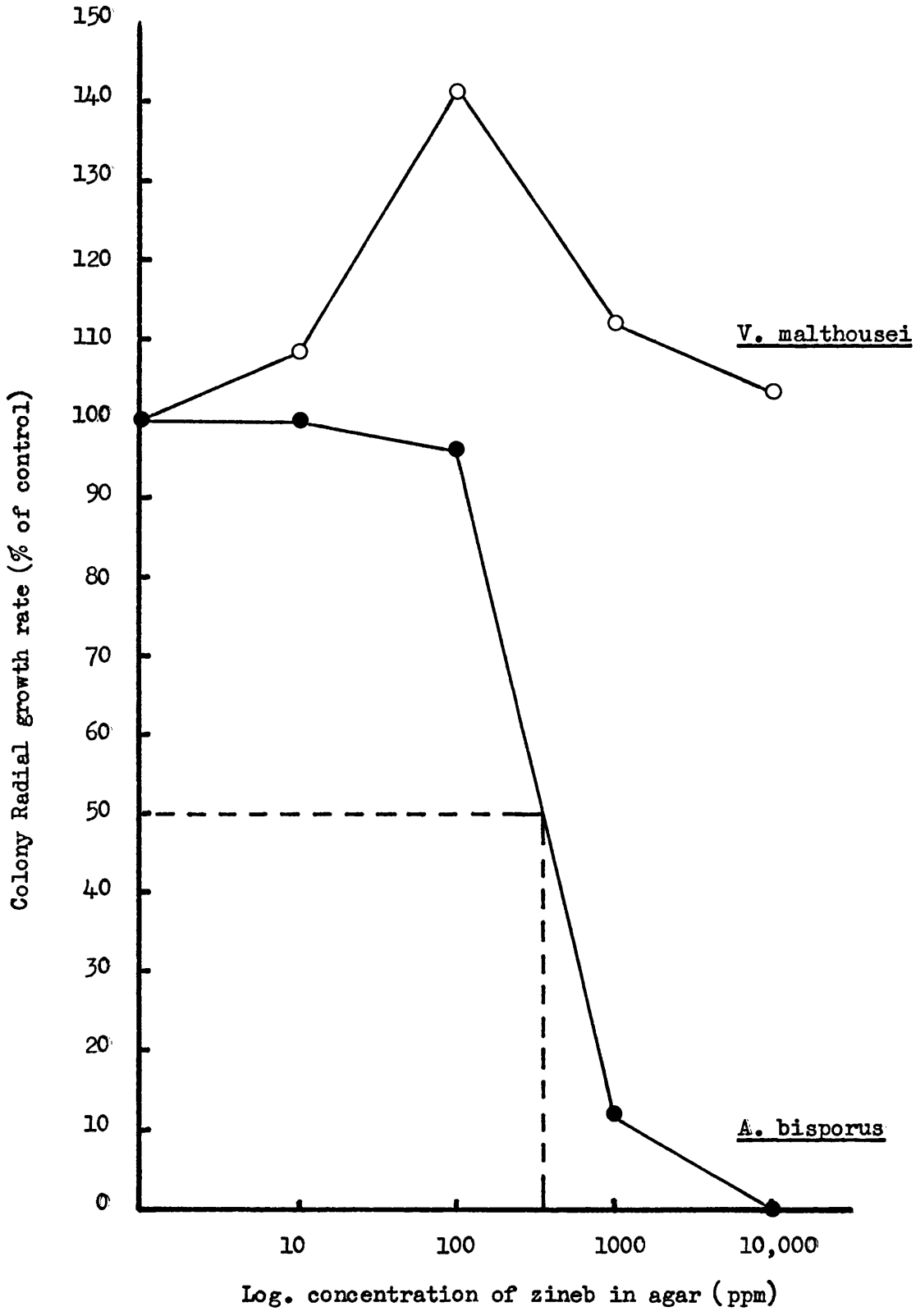


Fig. 19 Mean radial colony growth rates of A. bisporus and V. malthousei on malt agar containing zineb expressed as % of controls.

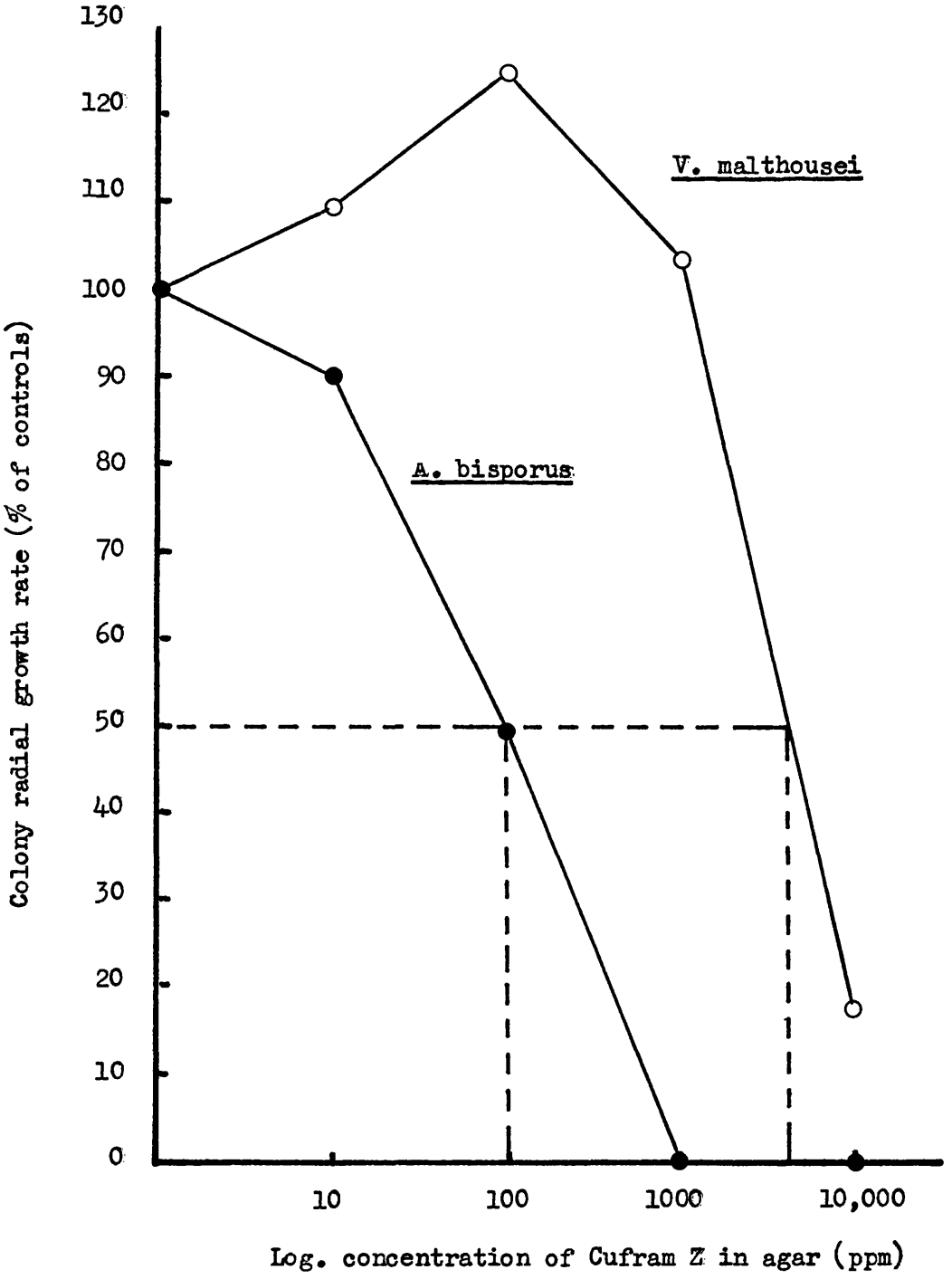


Fig. 20 Mean radial colony growth rates of A. bisporus and V. malthousei on malt agar containing Cufram Z, expressed as % of controls

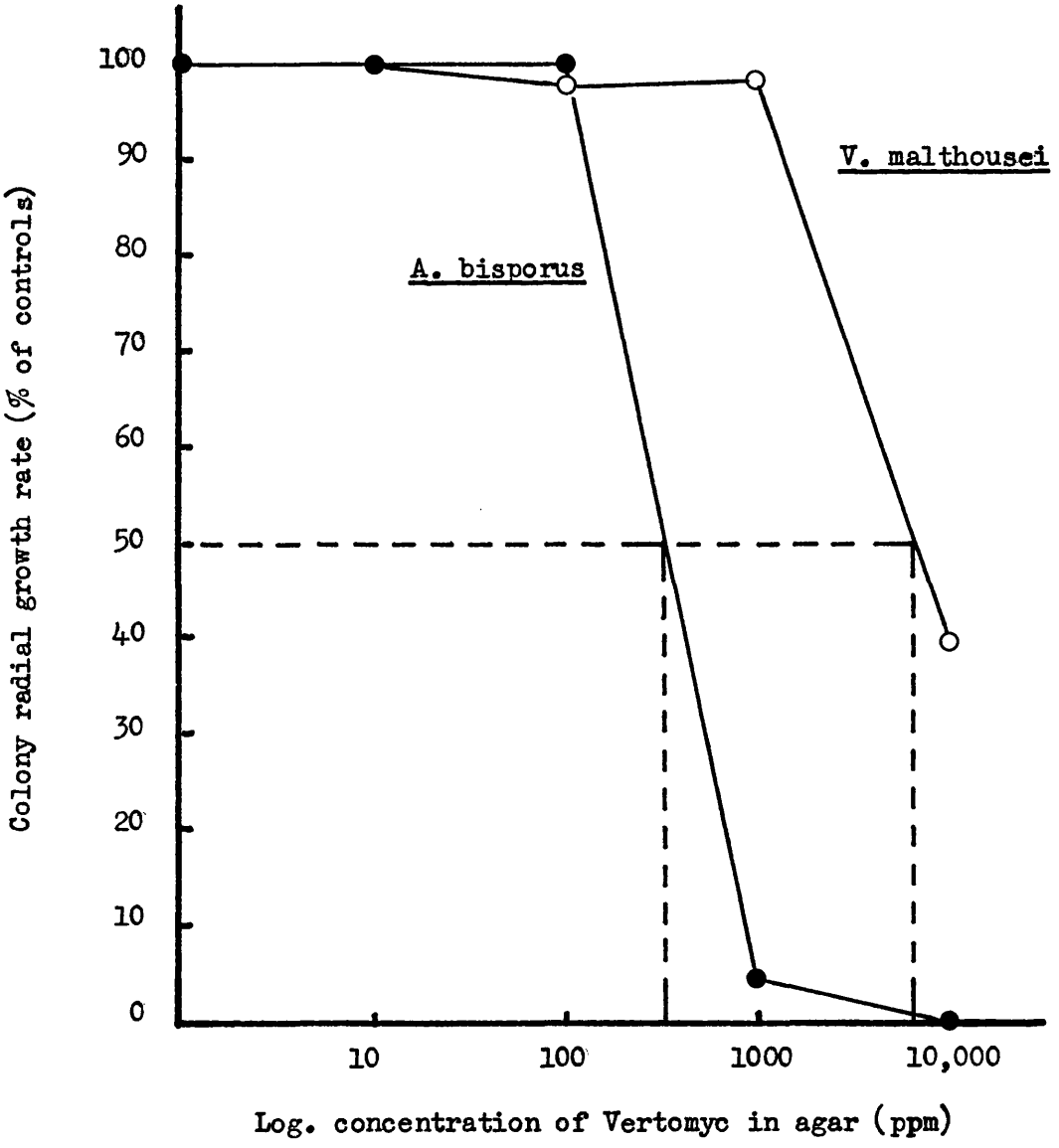


Fig. 21 Mean radial colony growth rates of A. bisporus and V. malthousei on malt agar containing Vertomyc, expressed as % of controls.

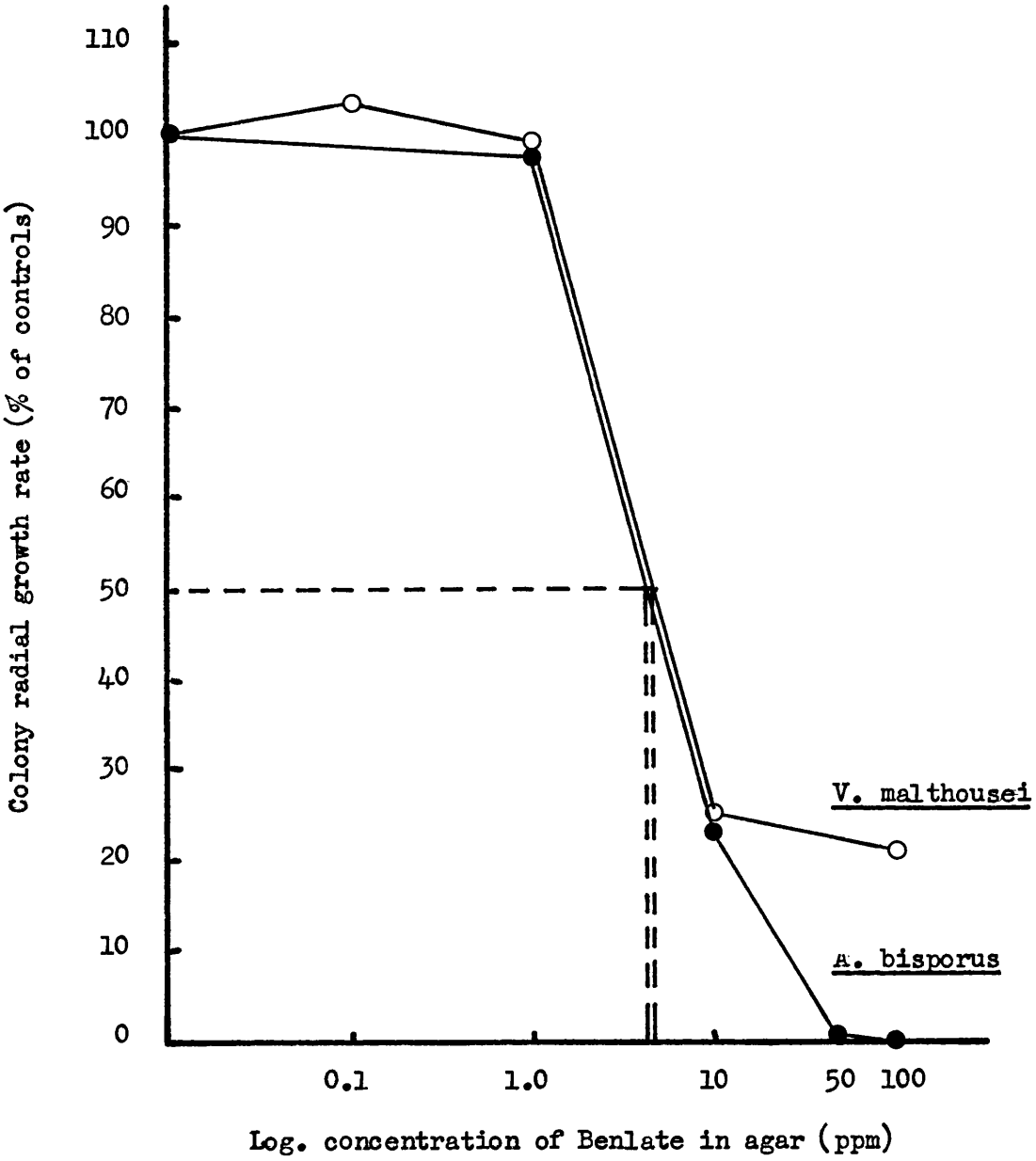


Fig. 22 Mean radial colony growth rates of A. bisporus and V. malthousei on malt agar containing Benlate, expressed as % of controls.

8. DISCUSSION

All *Verticillium* species are soil fungi and, with the exception of *V. lateritum* (Isaac 1967) are to a lesser or greater degree adapted to a parasitic mode of life. *V. albo-atrum* and *V. dahliae* cause vascular wilt symptoms in a wide range of economically important crops, but *V. nubilum* and *V. nigrescens* have a much more limited host range (Isaac 1949, 1953a, 1956) while *V. tricorpus* is confined to tomato, antirrhinum (Isaac 1953b) and possibly potato (Isaac 1967). *V. malthousei* appears to have a much more limited host range than other species of the genus and until recently was considered to parasitise *A. bisporus* only, but is now known to infect the hyphomycete *Rhopalomyces elegans* (Fletcher - personal communication) along with *V. psalliotae* Dayal and Barron 1970), *V. albo-atrum* and *V. dahliae* (Barron and Fletcher 1970). However, this parasitism occurred in mixed culture in the laboratory and has yet to be demonstrated in nature. If the natural occurrence of such parasitism is demonstrated, mycoparasitism would have great survival potential as suggested by Butler (1957) for *Rhizoctonia solani* and may also be a mechanism of increasing inoculum potential for plant pathogens in soil. The limited host range of *V. malthousei* is unusual, most mycoparasites having wider host ranges (Boosalis 1965), but is paralleled by *M. perniciosa* which is also adapted to infect *A. bisporus* although parasitism of *Rhopalomyces elegans* in

culture is known (Fletcher, personal communication). Another genus, Sepedonium, morphologically similar to Mycogone also contains species parasitic on basidiomycete sporophores.

Examination of the spore-producing structures of M. perniciosa suggests that the apparently xerosporic single conidia, delicately attached to the phialide tip appear to be well suited to wind dispersal. Thus it is surprising that spores of this organism are not dislodged directly from infected mushroom sporophores by wind, although indirect dispersal of spores of M. perniciosa and V. malthousei on wind-blown dust and debris, confirmed for the former by Fletcher and Ganney (1969), indicates that a filtered air supply to mushroom growing houses is necessary to prevent this method of disease transmission. Very little information on the dispersal of other fungi by this method has been reported (Hirst 1965) but a similar dissemination mechanism was described for V. albo-atrum following trapping of propagules twenty feet above ground on dust particles during a storm (Easton, Nagle and Bailey 1969). The gloeosporic conidia of V. malthousei would appear to be better suited to water and contact dispersal than spores of M. perniciosa due to the presence of mucilage (Mason 1937), a suggestion confirmed by the results of the present work. The implications for commercial growing practice are that diseased mushrooms should be covered (Jacobs 1965) prior to watering since rouging creates a further dispersal hazard. The control of fly populations is particularly

important in preventing dissemination of V. malthousei. Similar dispersal mechanisms operate for V. albo-atrum by water (Sewell 1959), contact (Keyworth 1942) and in crop debris (Isaac 1957, Kreitlow 1962).

V. malthousei differs from other species of Verticillium in that conidia survive at least eighteen months in soil and peat. V. albo-atrum and V. dahliae, producing resting mycelium and microsclerotia respectively in addition to conidia, did not survive a six month period in soil in the absence of a host. However, V. nubilum and V. nigrescens which produce chlamydospores and V. tricorpus producing resting mycelium, microsclerotia and chlamydospores, were recovered after twelve months (Isaac 1953a). Population fluctuations of the latter were attributed by Taylor (1969) to the production of short-lived conidia by microsclerotia in soil, a capacity also possessed by V. albo-atrum (Farley, Wilhelm and Snyder 1971). In contrast to V. malthousei the conidia of V. albo-atrum are short-lived, surviving in soil for only two to three weeks (Schreiber and Green 1962, Wilhelm 1965, Green 1969) but in staled culture conidia and microsclerotia survive three days and six months respectively (Nelson and Wilhelm 1958). The observation by Wilhelm (1955) that V. dahliae was recovered from soil fourteen years after the last known host crop had been planted was probably accounted for by results of Martinson and Horner (1962) and Lacy and Horner (1966) demonstrating production of new microsclerotia on roots of non-host plants.

V. albo-atrum appears unable to survive by producing resting mycelium on non-host roots which would account for its shorter survival in soil (Sewell and Wilson 1966). Whether V. malthousei can sporulate in soil in the presence of plant roots or fungi other than A. bisporus in the manner shown for microsclerotia of V. dahliae is not known, but such information would be of practical importance, particularly for American growers, many of whom use soil as a casing medium. The production of microsclerotia by V. malthousei has not been observed on infected mushrooms, in culture or in soil, but sporulation in soil associated with mushroom mycelium has been observed thus increasing its inoculum potential.

The survival experiment reported in the present work showed that conidia and immature chlamydospores of the mutant form of M. perniciosa became undetectable after only six months in peat casing material, their viability declining during this period. Inoculation of peat with conidia and chlamydospores of the parental M. perniciosa resulted in infection being maintained at an undiminishing rate for at least six months, the duration of the experiment. Assuming that conidia of M. perniciosa and the mutant have similar survival potential, this result indicates that M. perniciosa conidia survive in peat for a much shorter period than those of V. malthousei. Although not observed in pure culture, or on glass slides in contact with mushroom mycelium in casing material, germination of chlamydospores and subsequent mushroom infection

appears to be the only explanation of survival using mixed inocula indicating that long-term persistance of M. perniciosa is via chlamydospores. Thus the survival of V. malthousei and M. perniciosa for at least eighteen and six months respectively, poses great problems of mushroom farm site contamination, necessitating routine cleaning and disinfecting treatments to reduce this infection hazard.

The failure of the majority of V. malthousei conidia to germinate in soil while retaining their viability, is responsible for the survival of the organism - germ tubes and mycelium produced by the minority of spores soon undergoing lysis in the absence of a suitable host. The nature of this fungistasis (Dobbs and Hinson 1953), which is controversial, has been shown in the present study with V. malthousei, to be related to the requirement for spore germination, of a supply of exogenous nutrients, a feature of many other fungi subject to soil fungistasis (loc. cit.). Furthermore Ko and Lockwood (1967) showed for eighteen fungi a close correlation between sensitivity to soil fungistasis and germination capacity in distilled water in the absence of exogenous nutrients. Lysis of fungal mycelium in soil is induced by shortage of nutrients, antibiotics not being involved (Ko and Lockwood 1970). The capacity of nutrient independant spores of V. malthousei to germinate in soil, may be related to differences in storage reserves or to prior absorption of materials from the nutrient-contaminated spore suspension before being washed or added to soil.

Nutrient absorption for germination of other fungi under similar conditions has been shown to be cumulative and irreversible (Steiner and Lockwood 1969). Thus a requirement of exogenous nutrients for spore germination is the basis of the nutrient deficiency hypothesis of the nature of soil fungistasis proposed by Lockwood (1964).

A further requirement of this nutrient deficiency hypothesis is that fungistatic soil must be a nutritionally impoverished medium, with respect to materials which would allow spore germination to occur, caused by microbial competition for nutrients. Ko and Lockwood (1967) showed that extracts of sterilised soil contained sufficient nutrients for germination of nutritionally dependant spores while extracts of natural soil did not, a fact confirmed for V. malthousei in the present study, using the content of soluble hexoses as an index of nutritional status and supported by other workers who showed increases in easily extractable materials when soil is sterilised (Walker and Thompson 1949, Birch 1958, 1959, Dobbs 1963, Griffiths and Dobbs 1963). The fact that extracts of fungistatic soils are rarely inhibitory (Vaartaja 1969) and often stimulatory does not necessarily contradict the hypothesis of soil nutrient deficiency - a single localised source of nutrients in soil could obscure fungistasis in extracts by raising the concentration of extracted nutrients above the spore germination threshold level.

Assay methods have been one of the limiting factors in investi-

gations of soil fungistasis- direct incorporation of spores in soil involving the problem of recovery. This difficulty led to the development of semi-direct methods, where spores in contact with soil were supported on a surface, and indirect methods where spores are separated from soil by a semi-permeable membrane or assay medium - glass slides and plain agar discs respectively in the present study. Although the indirect agar disc methods of measuing soil fungistasis are less sensitive than the glass slide or 'cellophane' techniques (Jackson 1958, Dobbs, Hinson and Bywater 1960) they are considered comparable (Lockwood and Lingappa 1963), a fact confirmed by direct observation of fungal spores in soil (Ko 1971). Accepting the validity of the agar block technique in assaying soil fungistasis, a consequence of the nutrient deficiency hypothesis is the removal of the necessity to postulate the diffusion of a fungistatic factor into the block and to recognise that nutrients may diffuse out from the plain agar into soil, (Lockwood 1964) a possibility not previously considered. The use of a model system (Lockwood 1964, 1967) in the present work where diffusible nutrients were removed from plain agar blocks by dialysis, effectively reproduced the main features of soil fungistasis as assayed in agar blocks without any possibility of the presence of a fungistatic factor. This supporting evidence was confirmed by assaying the nutrient status of blocks exposed to soil and to dialysis. The results of the present study clearly indicate the controlling role of the level

of exogenous nutrients in spore germination of V. malthousei and although not disproving the existence of fungistatic factors, makes the postulation of such materials unnecessary in the mechanism of soil fungistasis.

Failure to appreciate the role of nutrients in soil fungistasis may lead to erroneous conclusions. Thus the claim by King and Coley-Smith (1969) that sclerotia of Sclerotium cepivorum, which are subject to soil fungistasis, germinate adequately in water may be invalidated by failure to ensure the absence of exogenous nutrients in the sterilised antibiotic assay discs used to determine germination in sterile distilled water. Results obtained from expressed soil solution which were not sterilised by membrane filtration must be disregarded because of the possible inhibitory action of soil micro-organisms in the extract (Lockwood 1964). Using assay discs soaked in expressates from fresh soils, sterilised by membrane filtration, 2 - 30% sclerotial germination was obtained compared with 12 - 32% germination, when water was used, for one isolate, the other being stimulated in comparison with controls. (King and Coley-Smith 1969). This gives little evidence for the conclusion drawn that inhibitory factors are responsible for soil fungistasis, rather supporting the nutrient deficiency hypothesis. However, using expressates from air-dried soils rewetted seven days before use, limited evidence of inhibitory materials was obtained. Similar conclusions were drawn by Hora and Baker (1970) who showed a declining effect of

volatile inhibitors resulting from microbial activity in rewetted air-dried soils over one to seven days, but this effect had disappeared fifteen days after rewetting. The fact that large quantities of nutrients become available in soil when rewetted after air-drying was demonstrated in the present work and by Birch (1958, 1959, 1960). The rapid growth of micro-organisms removing these or added nutrients is well documented (Chinn and Ledingham 1957, Boosalis 1962, Powelson and Patil 1963, Cook and Schroth 1965, Agnihotri and Vaartaja 1967, Bumbieris and Lloyd 1967, Cook and Flentje 1967, Dix 1967, Emmatty and Green 1967, Adams, Papavizas and Lewis 1968, Adams, Lewis and Papavizas 1968, Mircetich, Zentmeyer and Kendrick 1968, Adams and Papavizas 1969). However, the inability of King and Coley-Smith (1969) and Hora and Baker (1970) to demonstrate this in fresh field soil, and at fifteen days in rewetted soil respectively, indicates that the result of rewetting air-dried soil can be to modify the expression of soil fungistasis which occurs primarily in fresh soil and results from a dynamic equilibrium of microbial activity, maintaining a deficiency of materials required for spore germination in soil. A further type of spore germination inhibition observed in some soils has been distinguished from soil fungistasis in that it is not annulled by nutrients or sterilisation (Griffiths 1964, Dobbs and Gash 1965). In several reports, nutrient deficiency in soil has adequately accounted for the spore germination data obtained (Cooke and Schroth 1965, Powelson 1966, Cooke 1967,

Emmathy and Green 1967, 1969, Ko and Lockwood 1967, 1970, Adams, Lewis and Papavizas 1968, Emmathy 1969, Farley and Lockwood 1969, Mircetich and Zentmeyer 1969, Steiner and Lockwood 1969, 1970).

The effect of soil fungistasis on V. malthousei conidia in non-sterile soil and peat permits the long-term survival of nutrient dependant spores (Ko and Lockwood 1967) in this environment, the duration of which depends on the length of time the spore remains viable. Although susceptible to soil fungistasis (Powelson 1966) viability of conidia of V. dahliae and V. albo-atrum (Schreiber and Green 1963, 1966), is of short duration (Nelson and Wilhelm 1958, Green 1960, Schreiber and Green 1962, Wilhelm 1965). This indicates that survival of conidia in soil must be primarily determined by the inherent physiological factors controlling viability of spores, the germination of which can be influenced by soil fungistasis. V. dahliae conidia resemble those of V. malthousei in that exogenous nutrients are required for germination (Powelson 1966). The stimulatory effects of mycelial exudates for conidia of V. malthousei and of root exudates for conidia and resting structures of other species of Verticillium (Martinson and Horner 1962, Schreiber and Green 1963) and other genera of fungal plant pathogens (Cooke and Flentje 1967, Dix 1967, Adams, Papavizas and Lewis 1968, Mircetich, Zentmeyer and Kendrick 1968) illustrate the great significance of fungistasis in survival of soil-borne pathogens. Cochrane (1960) stated that 'an ecological advantage

may be conferred on a fungus which requires external nutrients in that spores will be protected from germinating in a milieu which is too impoverished to support mycelial growth'. Susceptibility to soil fungistasis and the dependance on host exudates for infection is for V. malthousei and other soil pathogens, an adaptation to a parasitic mode of life.

Apart from the growth of germ tubes from conidia adjacent to mushroom mycelium and the colonization of mushroom debris, the competitive saprophytic ability of V. malthousei as indicated by mycelial growth in unsterilised soil was shown to be very limited and is thus similar to V. albo-atrum and V. dahliae (Wilhelm 1951, Isaac 1953c, Sewell 1959, Green 1960, Wilhelm 1965, Huber and Watson 1970). V. malthousei may be classified as a 'host inhabitor' by analogy with classification of these two vascular wilt parasites in Garrett's (1956) category of 'root inhabitors', characterised by an expanding parasitic phase on the living host tissue and a declining saprophytic phase after its' death (Isaac 1967). V. nigrescens and V. nubilum were classified as 'soil inhabitors' characterised by an ability to survive indefinitely as soil saprophytes and that parasitism is incidental to their saprophytic existence in soil, while V. tricorpus was considered intermediate between these two types (Isaac 1967).

Direct comparison of the parasitism of V. malthousei with that of Verticillium species causing vascular wilts is difficult since these organisms attack the roots of higher plants. However,

neither the vascular wilt fungi nor the mushroom pathogen are limited to infection through the soil as V. albo-atrum may attack the above-ground parts of plants (Thanassoulopolous and Hooker 1970) in a similar manner to late infections by V. malthousei resulting in the cap-spotting syndrome. The parasitic status of Verticillium species causing vascular wilts was considered by Garrett (1956) to show affinity with primitive soil-inhabiting pathogens, due to their destructive invasion of immature tissues, their inability to invade the mature root cortex and their confinement within the vascular tract until the outer tissues of the root or shoot become monibund when sporulation on the surface may occur. V. malthousei shows more specialised parasitism in that it will infect mushrooms at any stage of growth - initials or mature sporophores, it is not restricted to particular areas of infected sporophores, it does not invade or breakdown the cells of the infected mushroom which remains living and growing for a considerable period and that V. malthousei is able to sporulate on the external surfaces of the living infected sporophore. No evidence has been obtained for the pathogen causing a pre-emergence destruction of initials. Thus it is concluded that in comparison with the organisms causing vascular wilts of higher plants, particularly V. albo-atrum and V. dahliae, the parasitism of V. malthousei is relatively specialised.

In comparison with other mycoparasites, using the classification of Boosalis (1965), V. malthousei is a 'biotrophic' parasite obtaining nutrients from living cells by hyphae in close contact

with host cells rather than by haustoria as in Piptocephalis sp. (Dobbs and English 1954, Leadbeater and Mercer 1957). In contrast 'necrotrophic' fungi obtain their nutrition from dead cells, usually killing the host prior to infection (Barnett 1959). The tropic response of conidia of V. malthousei, stimulated to germinate and growing toward host mycelium is similar to that observed in Piptocephalis virginiana (Berry and Barnett 1957) while in Calcarisporium parasiticum the mycelium of the host Physalospora sp. grows towards the pathogen (Barnett and Lilly 1958). A contact stimulus appears to be operative in Rhizoctonia solani which can be induced to respond thigmotropically to glass fibres (Butler 1957).

'Balanced' biotrophic mycoparasites cause little or no damage to the host, 'destructive' biotrophic parasites killing the host during their development (Boosalis 1965). Since V. malthousei is able to parasitise the mushroom sporophore but not vegatative mycelium growing in compost, it would appear to fall between these two extremes as the destructiveness of the disease produced in a sclerodermoid mushroom is manifest in the lack of tissue differentiation. Information on the viability of cells of infected mushroom sporophores would enable a more informed appreciation of the nature of the parasitism of V. malthousei to be made. The parasitism of M. perniciosa while similar in many respects to that of V. malthousei differs in that host cell breakdown, death and putrefaction rapidly occurs, so the parasite is considered less specialised

than V. malthousei.

The in-vivo work on the use of fungicides to control disease caused by V. malthousei confirmed earlier reports (Gandy 1957, Last and Gandy 1965) that the protective use of the commercial preparations of zineb used on mushroom beds is ineffective against the level of inoculum used at the fungicide application rate recommended by Sinden and Yoder (1949). Using the single application of fungicide, no reduction in the numbers of healthy mushrooms was observed as fungicide concentrations increased, some stimulation being noted, in contrast to the reports of Yoder et al (1950) and Philipp (1963) where more than one application was used. Inoculation of the casing three days after treatment with zineb resulted in a consistently higher infection rate but also required a tenfold increase in fungicide concentration to achieve a reduction in numbers of diseased mushrooms. This latter result suggests that the efficiency of the fungicide has become reduced in the interval and underlines the ineffectiveness of this materials, as fungicide treatment relative to infection cannot be synchronised under commercial conditions. Although zineb is reported to be unstable in water (Anon 1959) this reduction in efficiency in disease control is unlikely to be due to instability as this material was shown in the present study to be relatively stable in water and soil over a period of six and fourteen days respectively. This finding is confirmed by Richardson (1971) who in a chemical analysis of soil using an adaptation of

the methods of Clarke, Baum, Stanley and Hester (1951) and Cullen (1964) showed zineb to be stable compared with a 45% loss of maneb and an 84% loss of nabam in both sterile and natural soil over a period of seven days.

The work reported earlier with V. malthousei and zineb supports the view of Horsfall and Lukens (1966) that dithiocarbamates do not induce resistance in fungi. Thus the lack of effectiveness of zineb in mushroom disease control cannot be attributed to the development of resistance in V. malthousei.

The assay of zineb in soil with spores of V. malthousei supported on glass slides indicated that the standard application rate (Sinden and Yoder 1949) inhibitory if confined to the surface would also be effective at preventing spore germination if distributed through approximately 1.3 mm. Burgess (1950) showed that mucilagious spores washed readily downwards through soil, but no data on the penetration of zineb into casing materials have been produced. In the disease control experiment where zineb was applied at one hundred times the standard rate, the fungicide may be distributed throughout the casing and remain above the minimum concentration which prevented spore germination in the soil assay, yet still failing to give complete disease control. However, in contrast to the soil assay when a glass surface was used, when spores are sprayed on casing materials they are distributed over an large surface area of particles or fibres. With a largely insoluble fungicide it is possible that this, together with physical distri-

bution of the fungicide particles in casing which may produce areas of poor fungicide coverage within the casing, results in a lack of effectiveness at preventing spore germination. This concept is reinforced by the lack of toxicity of such applied zineb to mushroom mycelium indicated by the absence of reduced numbers of mushroom sporophores growing where high zineb concentrations were applied. In addition, if leaching removes some of the fungicide, some previously inhibited spores may germinate due to the sporostatic effect of zineb at a limited range of concentrations.

The ability of V. malthousei mycelium to grow at high concentrations of the dithiocarbamates tested and the increased growth rates induced by zineb and Cufram Z, together with the relative susceptibility of A. bisporus mycelium to these fungicides, may be a further factor in the previously demonstrated inefficiency of zineb to control mushroom disease caused by V. malthousei. The greater toxicity of Cufram Z and Vertomyc may be associated with their manganese content as it has been shown (Ludwig, Thorn and Unwin 1955, Ludwig and Thorn 1958), that manganese ions catalyse the oxidation of dithiocarbamates and a breakdown product, isothiocyanate to ethylene thiuram monosulphide, both proposed as more toxic metabolites of dithiocarbamate fungicides (Klopping 1951, Ludwig and Thorn 1953, Vonk and Sijpesteijn 1970). The germination of V. malthousei conidia is inhibited in water and on agar by similar concentrations of zineb indicating that the

large difference in response to the fungicide between spore germination and mycelial growth is not due to the use of different methods. Thus support is given to the observation by McCallan and Miller (1957) that germinating spores accumulate fungicide more rapidly than mycelium. However, the difference in sensitivity is so large that it may be due to the use of quite different metabolic routes in the physiology of spore germination and mycelial growth. It has been shown that sensitivity to PCNB in Rhizoctonia (Corticum) solani compared with Fusariun salani was due to a detoxication mechanism rather than differing absorption rates (Ko 1968).

The greater toxicity of benomyl to mycelial growth of V. malthousei than A. bisporus obtained by Wuest and Cole (1970) and Snel and Fletcher (1971) was not demonstrated in the present work possibly because of V. malthousei stain differences in tolerance to this material (Wuest and Cole 1970). In addition a different method was used, dissolving the fungicide in acetone prior to addition to agar (Snel, Schmeling and Edgington 1970) while in the present work it was added to agar as an aqueous suspension, as it would be used in soil. This may result in the formation of a new compound differing in toxicity to the original benomyl as shown by Clemons and Sisler (1969) who found that in aqueous solution benomyl was transformed to benzimidazole carbamic acid methyl ester, both compounds equally toxic to Neurospora crassa and Rhizoctonia (Corticum) solani but benomyl was thirty times more

toxic to Saccharomyces pastorianus.

Control of mushroom disease caused by M. perniciosa which has a similar response to benomyl to V. malthousei (Snel and Fletcher 1971) has been demonstrated in laboratory tests and in commercial practice following incorporation of benomyl in the casing (Snel and Fletcher 1971, Drakes and Fletcher 1971). Application of benomyl to the casing surface has given control of V. malthousei (Gandy 1971). However, Hine, Johnson and Wenger (1969) showed that significant movement of benomyl in glasshouse soils does not occur and it was detectable twenty two weeks after application, while this material has been recovered from mushroom sporophores after six weeks of cropping following incorporation in casing (Drakes and Fletcher 1971). Stimulation of mycelial growth of another basidiomycete by benomyl has been reported by Smith, Stynes and Moore (1970). Benomyl contains an imidazole group structurally related to other imidazole compounds which are precursors of purine bases (adenine, guanine) in the biosynthesis of nucleoside triphosphates and nucleic acids (Mahler and Cordes 1966). Thus the possibility exists that if absorbed and metabolised, benomyl could provide analogue purine base components the incorporation of which may have genetic consequences. Hastie (1970) showed that benlate induced the formation of segregants in Aspergillus nidulans and considered that it may operate directly on chromosome segregation or act as a mutagen causing chrosome breakage. Thus the possibility of genetic effects in man following consumption of mushrooms containing benomyl or a fungitoxic

decomposition product (Clemons and Sisler 1969, Peterson and Edgington 1970) must introduce caution into the use of this material in agriculture generally or more particularly in mushroom disease control.

It is interesting to note that Wuest and Cole (1970) have demonstrated variable responses to benomyl using V. malthousei. Resistance to this material has been reported for Sphaerotheca fruliginea, causal organism of powdery mildew of cucurbits, (Schroeder and Provvidenti 1968, 1969) suggesting that this fungicide is highly specific in its mode of action, a property which could severely limit its usefulness.

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APPENDIX I

Composition of Mushroom Extract Media

Mushroom extract medium A was a macerate of 40 g. fresh mushrooms in one litre of distilled water with 15 g. agar. Medium B was similar but with 15 g. malt extract added. Both A. and B. were sterilised in the autoclave at 10 lbs/in^2 for ten minutes. Medium C. is a membrane-filtered water extract of macerated mushroom tissue mixed with a equal volume of molten, cooled 3% agar when poured.

APPENDIX II

46

Table (part 1) Germination of *V. malthousei* conidia on dialysed plain agar blocks treated with glucose solutions after incubation on soil

Period on soil (hr)	Glucose conc.(ppm)	No. spores germinated	No. spores ungerminated	Percentage germination	Mean % germination
0	0	53	181	22.6	22.0
		46	170	21.3	
	0.01	76	132	36.5	31.1
		53	153	25.7	
	0.1	60	156	27.8	19.5
		25	198	11.2	
	1.0	48	147	24.6	24.7
		56	171	24.7	
	10	88	164	34.9	54.1
		176	64	73.3	
	100	145	141	50.7	60.3
		180	78	69.8	
	1000	123	115	51.7	61.7
		166	66	71.6	

Table V46(part 2) Germination of *V. malthousei* conidia on dialysed plain agar blocks treated with glucose solutions after incubation on soil

Period on soil (hr)	Glucose conc.(ppm)	No. spores germinated	No. spores ungerminated	Percentage germination	Mean % germination
8	0	69	207	25.0	21.8
		41	179	18.6	
	0.01	32	175	15.5	17.0
		45	199	18.4	
	0.1	133	136	49.4	34.8
		43	171	20.1	
	1.0	85	124	40.7	35.5
		67	154	30.3	
	10	240	93	72.1	56.3
		94	141	40.0	
	100	207	39	84.1	74.5
		157	85	64.9	
	1000	167	37	81.9	76.0
		152	65	70.0	

Table 46(part 3) Germination of *V. malthousei* conidia on dialysed plain agar blocks treated with glucose solutions after incubation on soil

Period on soil (hr)	Glucose conc.(ppm)	No. spores germinated	No. spores ungerminated	Percentage germination	Mean % germination
16	0	44	169	20.7	18.4
		35	183	16.1	
	0.01	67	145	31.6	34.4
		79	134	37.1	
	0.1	32	173	14.9	22.2
		82	197	29.4	
	1.0	112	102	52.3	43.3
		83	159	34.3	
	10	176	34	83.8	71.2
		153	108	58.6	
	100	181	65	73.6	72.0
		195	71	70.7	
	1000	169	54	75.6	75.2
		189	64	74.7	

Table 46(part 4) Germination of *V. malthousei* conidia on dialysed plain agar blocks treated with glucose solutions after incubation on soil

Period on soil (hr)	Glucose conc.(ppm)	No. spores germinated	No. spores ungerminated	Percentage germination	Mean % germination
24	0	7	201	3.4	19.9
		99	174	36.3	
	0.01	55	189	22.5	31.7
		87	126	40.8	
	0.1	111	190	36.9	32.0
		65	176	27.0	
	1.0	86	221	28.0	35.4
		123	165	42.7	
	10	126	77	62.1	53.4
		104	129	44.6	
	100	177	40	81.6	83.9
		181	29	86.2	
	1000	193	45	81.1	84.7
		315	42	88.2	

A P P E N D I X III

Table 47 (part 1)

Mean colony diameters of *V. malthousei* and *A. bisporus* grown on malt agar containing fungicide

Organism / Fungicide	Fungicide conc. (ppm)	Mean colony diameter (mm) Day 0	Day 3	Day 6	Day 9
V. malthousei	0	12.0	17.0	21.8	26.4
Zineb	10	11.5	17.2	22.4	27.4
	100	9.2	16.0	23.0	30.1
	1000	7.6	12.8	18.0	23.8
	10000	7.0	11.7	17.8	22.2
A. bisporus	0	16.9	29.4	41.3	53.6
Zineb	10	16.9	29.4	40.8	53.6
	100	12.1	23.1	36.2	47.8
	1000	4.7	5.8	7.0	9.4
	10000	3.0	3.0	3.0	3.0
V. malthousei	0	11.3	16.4	21.2	25.6
Cufram Z	10	9.7	15.4	20.8	25.2
	100	8.1	13.7	20.1	26.1
	1000	5.6	9.8	15.0	20.7
	10000	5.2	6.6	7.1	7.6
A. bisporus	0	15.4	28.1	40.3	51.7
Cufram Z	10	11.8	22.2	33.7	44.4
	100	7.8	14.0	19.7	23.3
	1000	3.0	3.0	3.0	3.0
	10000	3.0	3.0	3.0	3.0

Table 47. (part 2)

Organism Fungicide	Fungicide conc. (ppm)	Mean colony diameter (mm) Day 0	 Day 3	 Day 6	 Day 9
V. malthousei	0	10.8	16.1	21.0	25.4
Vertomyc	10	10.9	16.1	20.9	25.8
	100	10.0	14.7	19.5	24.9
	1000	5.1	10.2	14.8	20.0
	10000	4.4	5.9	8.3	10.7
A. bisporus	0	16.0	28.6	39.7	52.1
Vertomyc	10	14.8	27.6	38.9	51.6
	100	10.9	18.9	32.8	42.7
	1000	4.2	5.1	6.0	6.2
	10000	3.0	3.0	3.0	3.0
V. malthousei	0	12.0	17.0	21.8	26.4
Benlate	0.1	12.4	17.7	21.7	27.2
	1.0	11.8	16.8	21.5	25.4
	10	4.5	5.7	7.0	8.2
	100	4.6	5.4	6.7	7.6
A. bisporus	0	16.9	29.4	41.3	53.6
Benlate	1	15.2	27.0	39.2	51.4
	10	8.0	11.2	12.9	16.2
	50	3.0	3.1	3.2	3.2
	100	3.0	3.0	3.0	3.0

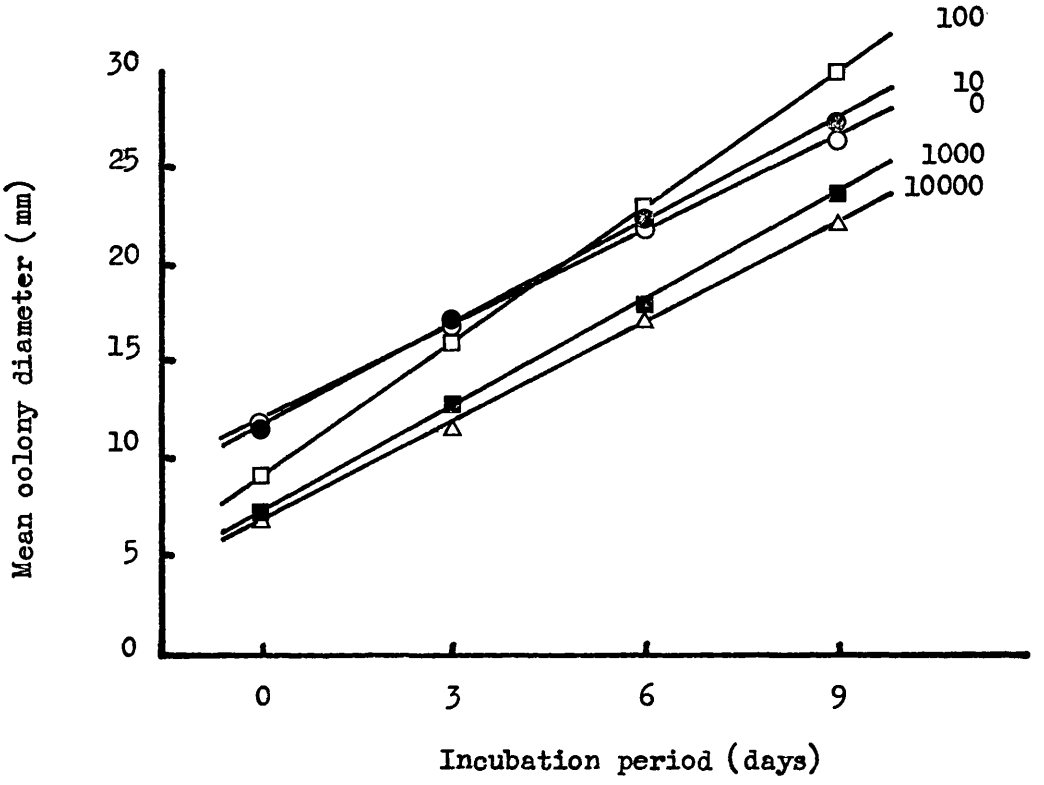

Fig. 23 Growth of V. malthousei on agar containing zineb

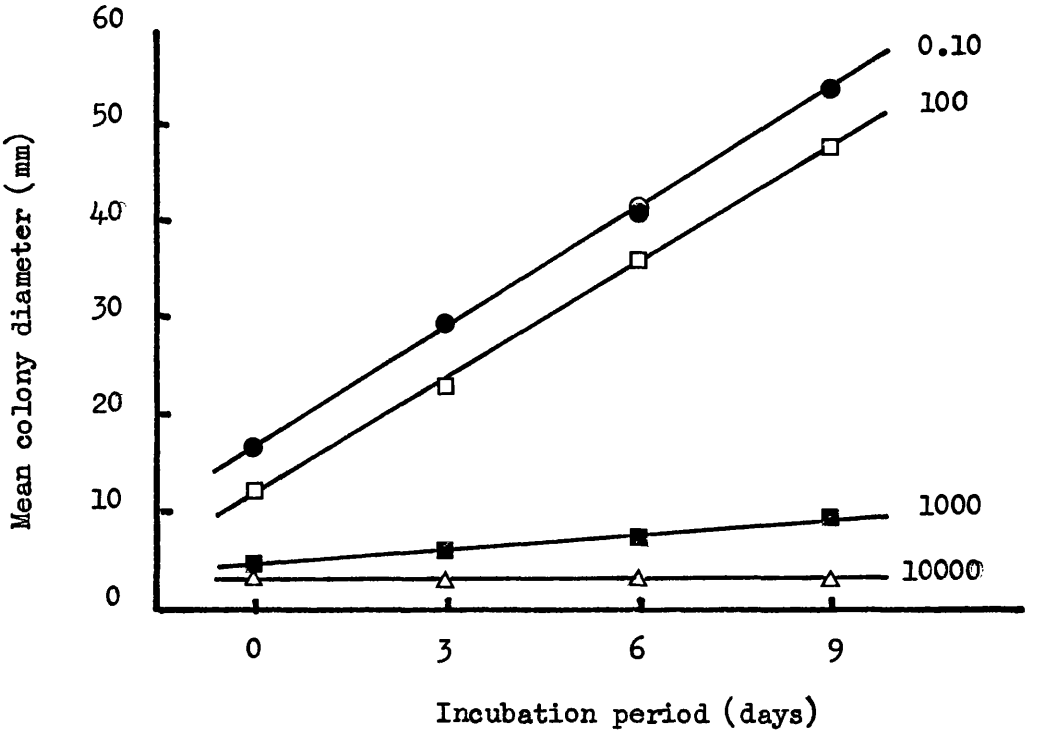

Fig. 24 Growth of A. bisporus on agar containing zineb

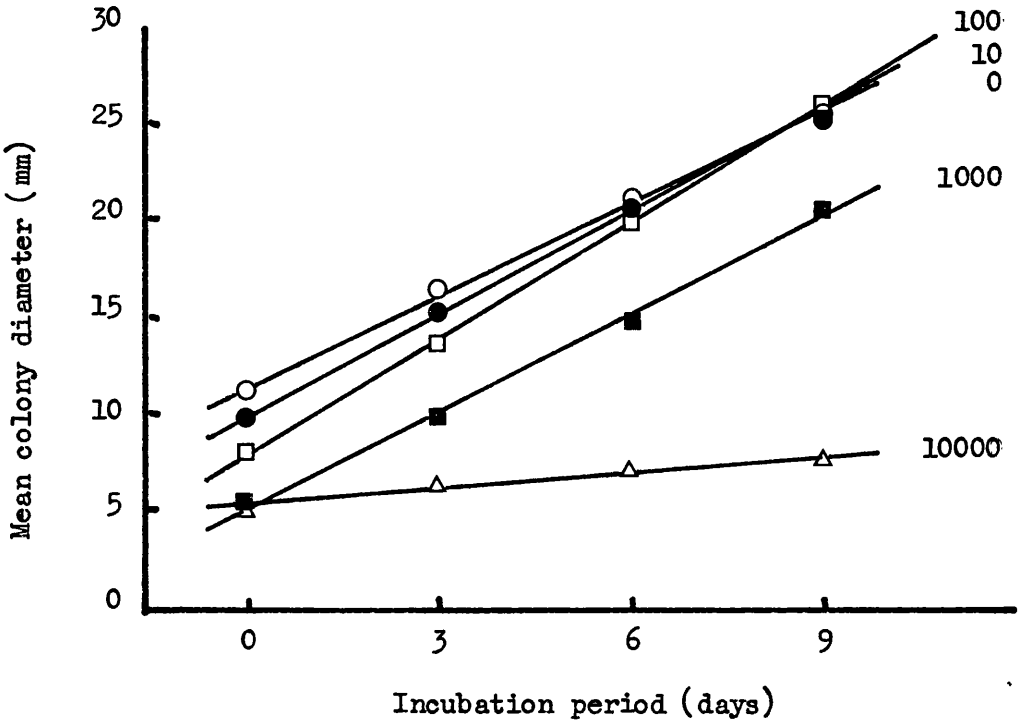

Fig. 25 Growth of V. malthousei on agar containing Cufram Z

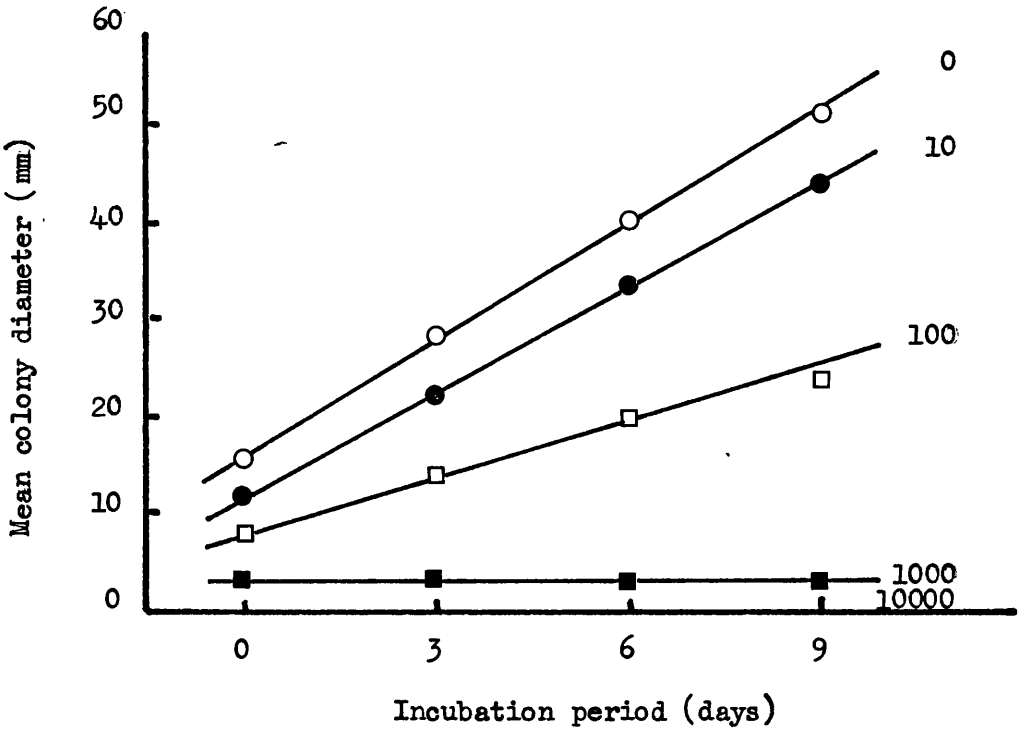

Fig. 26. Growth of A. bisporus on agar containing Cufram Z

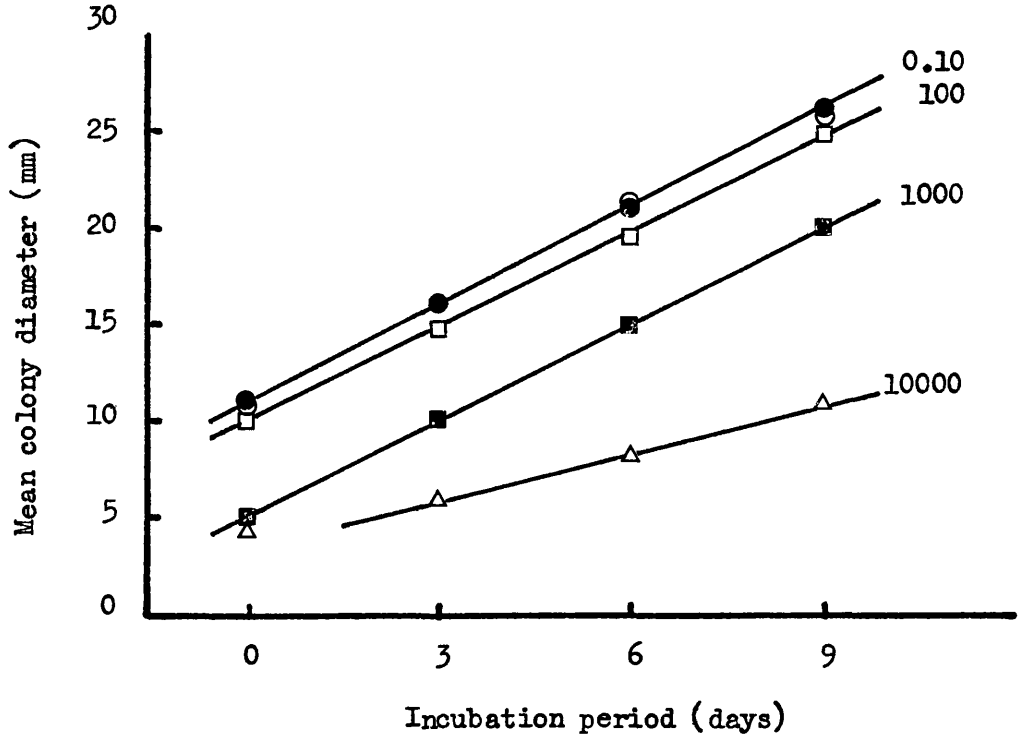

Fig. 27 Growth of V. malthousei on agar containing Vertomyc

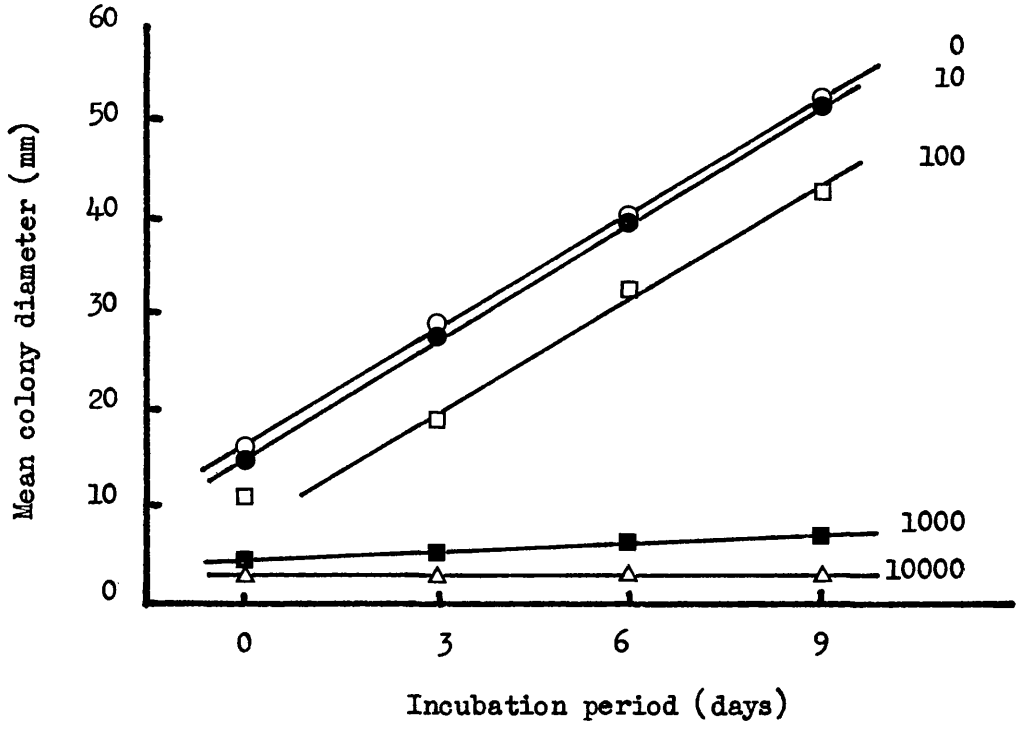

Fig. 28 Growth of A. bisporus on agar containing Vertomyc

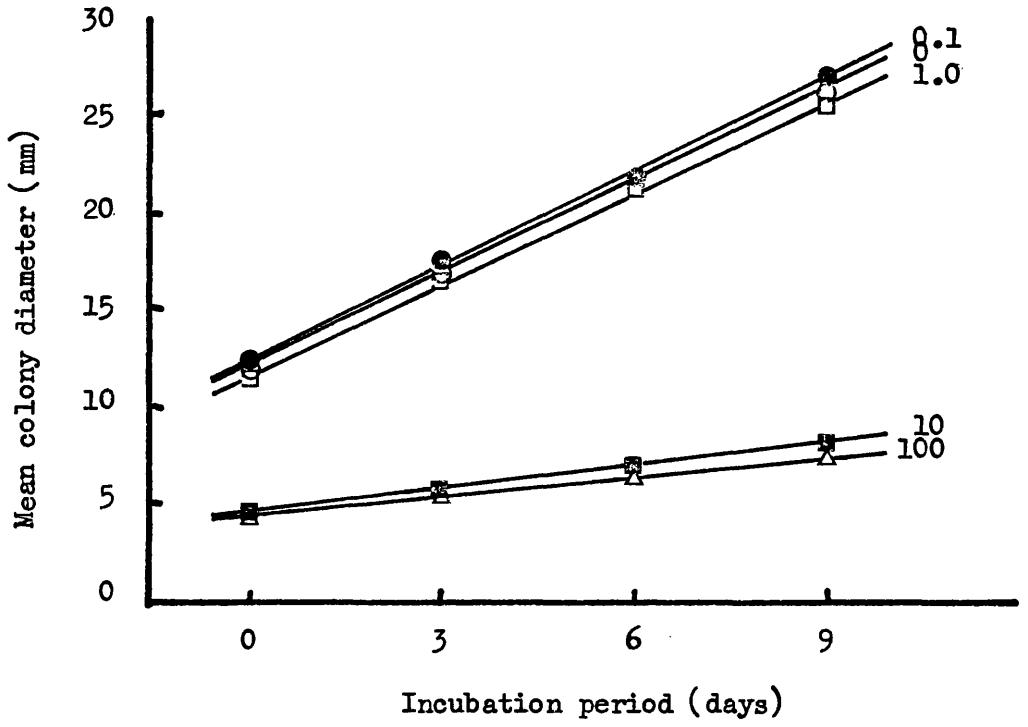

Fig. 29 Growth of V. malthousei on agar containing Benlate

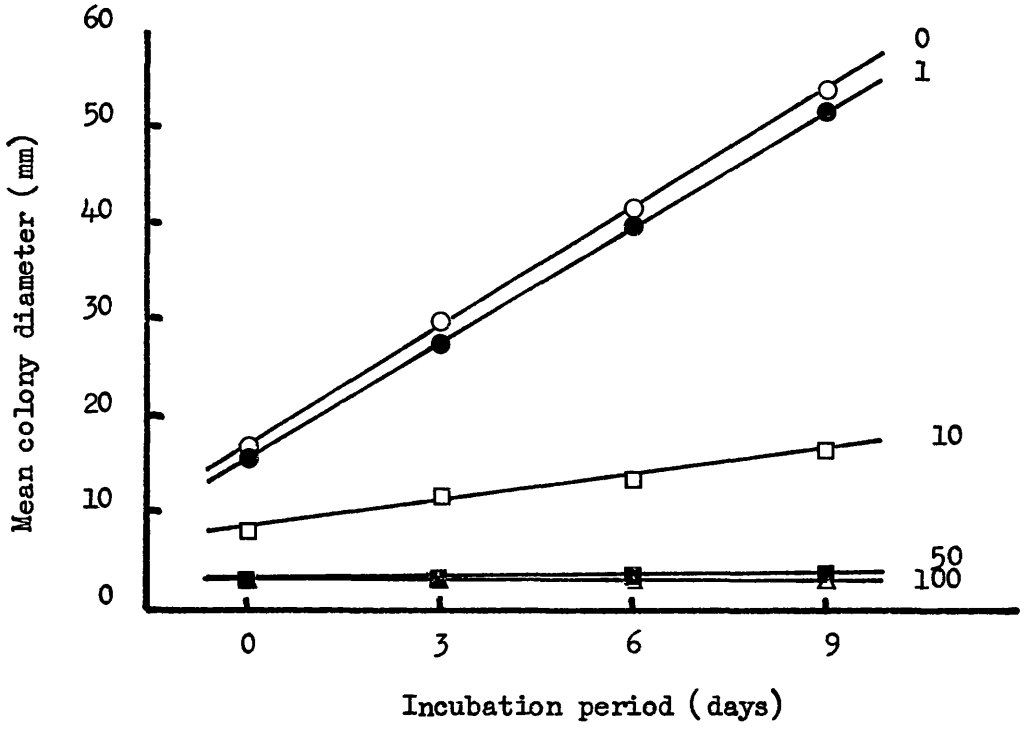

Fig. 30 Growth of A. bisporus on agar containing Benlate